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# CHROMATOGRAPHY

*By*

HAROLD G. CASSIDY, NORMAN APPLEZWEIG, STIG CLAESSON, VICTOR R. DEITZ, BEVERIDGE J. MAIR, A. J. P. MARTIN, STANFORD MOORE, ROBERT L. PECK, W. A. SCHROEDER, LEO SHEDLOVSKY, WILLIAM H. STEIN, HENRY C. THOMAS, AND L. ZECHMEISTER

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# INTRODUCTION TO THE CONFERENCE ON CHROMATOGRAPHY

*By* Harold G. Cassidy

*Sterling Chemistry Laboratory, Yale University, New Haven, Connecticut*

The conference which opens this morning is another in the series sponsored by the Section of Physics and Chemistry of The New York Academy of Sciences. The subject of this conference is *chromatography,* and the papers to be presented will deal with the scope, theory, manipulation, and application of this tool.

Dr. Fruton suggested about a year ago that a conference on this subject be arranged. It was felt that it would be profitable to examine the state of our knowledge regarding chromatography, and especially to make it possible for workers using one method of adsorption analysis to come together with those using other methods, so that all methods might become more widely known. It was hoped that, through such a meeting, the chromatographic tool might become further sharpened and the realm of its proper use redefined.

This present conference developed from these ideas. It emphasizes the very fundamental fact that the improvement of an analytical tool is of major importance to the science, for many an improvement in analytical technique has changed the face of scientific theory. This conference recognizes that the labor of the analyst is as endless as it is rewarding. One thinks, in this connection, of Walt Whitman's lines, "For it is provided in the essence of things, that from any fruition of success, no matter what, shall come forth something to make a greater struggle necessary." No sooner has one problem been solved than the very solution of it raises others of greater subtlety. We have gathered here to examine the new demands which success imposes, as well as to enjoy some of its fruits.

We have met here to discuss chromatography. It is only one of many analytical tools. Like so many of the procedures used by chemists to separate mixtures, it depends upon a partition process. In it, the substances to be separated are allowed to distribute themselves between two or more phases, and the operation is completed by a mechanical separation of the phases. In the case of chromatography, the partition occurs between a fluid phase and an interfacial phase. It is, in short, an adsorption process, and this distinguishes chromatography from other separation processes. In another respect, however, chromatography is similar to other separation methods, such as fractional distillation under reflux, countercurrent extraction, and countercurrent crystallization, in that advantage is taken of the partition in the most efficient manner possible, namely through a countercurrent application. It is partly in this respect that partition chromatography belongs here. It is worth emphasizing, perhaps, on the basis of these similarities, that much that will be said in this publication has implications beyond chromatography.

In the spirit of these Conferences, only relatively few papers have been arranged for, so that there will be ample time for pertinent discussion after each paper. This has meant that many interesting subjects (chiefly specific applications) have had to be passed over. However, we have tried to obtain a balance between theory and method on the one hand and application on the other.

It is now time to turn to those experts who, during the conference, will act as our guides, philosophers, and friends. They will guide us by paths many of which they have themselves broken, and they will discover for us, in the discussion of chromatography, principles which are at the foundation of all analysis and, hence, belong to the very philosophy of science. It is hoped that out of this meeting will come the stimulus to many new researches, hypotheses, and discoveries in and through the use of chromatography.

# HISTORY, SCOPE, AND METHODS OF CHROMATOGRAPHY

*By* L. Zechmeister

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## HISTORY

It is one of the characteristic features of the history of organic chemistry that the direction and rate of progress have depended, to a marked extent, upon the availability and effectiveness of physical methods. In the eighteenth century, when the chemistry of the carbon compounds began to emerge from its infancy, crystallization played an important role in laboratory technique and soon developed into fractional crystallization. Since the middle of the nineteenth century, distillation, an art practised for thousands of years, has gradually developed into fractional distillation of very high efficiency without which modern developments in the field of volatile substances would not have been possible. Likewise, in the present century, we are witnessing the development of the adsorption process into manifold methods of fractional adsorption and elution without which a deeper study of many non-volatile compounds would be unthinkable.

While the history of crystallization or distillation cannot be traced to a unique source, chromatography is based on a single principle and is the invention of one individual: the principle is the passing of a solution through an adsorbing column and the inventor is Michael Tswett.

Tswett possibly was influenced by the work of Goppelsroeder, whose experiments on capillary analysis he cites in one of his first papers. Goppelsroeder first described this subject as early as 1861 and, much later, in his well known monograph (1901). The basic observations were, however, made by Goppelsroeder's teacher, Schoenbein (1861, 1864) who observed selective adsorption of the components of a mixture by the different heights to which they rose when a strip of filter paper was dipped into the solution. Schoenbein made his first observations during his classic studies of ozone, when he impregnated paper with potassium iodide-starch or other ozone reagents.

At the age of 34, Tswett, who was the son of a Russian father and an Italian mother, described the fundamental principle and technique of chromatography in an eight-page article (1906) which reached the Editors of the *Berichte der deutschen botanischen Gesellschaft* on June 21, 1906, a date which we may accept as the official birthday of chromatography. In this remarkable paper he tells the story of his discovery.

It was known that various solvents behave very differently in the manner in which they extract the pigment of carefully dried green leaves. Alcohol or ether removes all of the pigments and, in such an extract, the dark green color of chlorophyll obscures the yellow carotenoids. However, petroleum ether yields a yellow extract which contains chiefly carotenoids and is practically free of chlorophyll, in spite of the fact that crude chlorophyll samples, once prepared, are easily soluble in petroleum ether. Tswett also observed that, if as little as 1 per cent of alcohol is added to the petroleum ether, the entire chlorophyll of the leaves goes into solution. He asks the question: "What is the solubility-promoting effect of the alcohol?"

In order to gain some experimental data, Tswett prepared a petroleum ether-alcoholic total extract into which he placed filter paper strips, whereupon the solvent was evaporated. The dried dark-green strips behaved exactly like the leaf powder: pure petroleum ether gave a yellow extract, but the same solvent containing some alcohol yielded a dark green solution and the paper became colorless. Therefore, Tswett concluded:

"The previously puzzling phenomena which were mentioned at the beginning of this article are, consequently, based on the adsorption of the pigment . . . which is overcome by alcohol, ether, etc. but not by petroleum hydrocarbons."

Being a man of imagination, Tswett, forty years ago, developed an astonishingly clear concept of the fundamental processes upon which modern chromatography is based. He wrote:

"An adsorbent which is saturated with one substance is still able to take up and bind a certain amount of another. However, then also substitutions may take place. For example, the xanthophylls are partially displaced by the chlorophyllins from their adsorption compounds; but not *vice versa*. There exists a certain adsorption sequence, according to which the substances are able to replace one another. The following important application is based on this law. If a petroleum ether solution of chlorophyll is filtered through a column of an adsorbent (I use mainly calcium carbonate which is stamped firmly into a narrow glass tube), then the pigments, according to the adsorption sequence, are resolved from top to bottom into various colored zones, since the stronger adsorbed pigments displace the weaker adsorbed ones and force them farther downwards. This separation becomes practically complete if, after the pigment solution has flowed through, one passes a stream of pure solvent through the adsorbent column. Like light rays in the spectrum, so the different components of a pigment mixture are resolved on the calcium carbonate column according to a law and can be estimated on it qualitatively and also quantitatively. Such a preparation I term a chromatogram and the corresponding method, the chromatographic method.

"It is self-evident that the adsorption phenomena described are not restricted to the chlorophyll pigments, and one must assume that all kinds of colored and colorless chemical compounds are subject to the same laws. I have so far investigated lecithin, alkannin, prodigiosin, sudan, cyanin, solanorubin, and acid derivatives of the chlorophyllins with positive success."

Evidently, Tswett is the true inventor of chromatography in all of its important aspects. However, when the time is opportune for the clear formulation of a new principle, several minds may simultaneously have the information on which to base it. In this country, thousands of miles from Tswett's laboratory and nine years before him, Day (1897), discussing the origin of Pennsylvania earth oil, wrote:

". . . by experimental work it may easily be demonstrated that if we saturate a limestone, such as the Trenton limestone, with the oils characteristic of that rock and exert slight pressure upon it, so that it may flow upward through finely divided clay, it is easy to change it in its color to oils similar in appearance to the Pennsylvania oils, the oil which first filters through being lightest in color and the following oils growing darker."

Later, Day induced Gilpin and his collaborators (1908, 1910, 1913) to carry out detailed experiments, and they showed, evidently without the knowledge of Tswett's papers, that, if crude oil is forced upward through a column of fuller's earth, the following sequence can be noticed from top to bottom: saturated aliphatic hydrocarbons, then aromatics and unsaturated substances, and, finally, nitrogen and sulfur compounds, the amounts of which increased towards the bottom "because of selective adsorption."

These experiments, originating from considerations which were quite different from Tswett's, might well, under favorable conditions, have developed into systematic chromatography.

As is well known, the significance of an outstanding discovery is not always understood immediately. In many cases, further developments begin only after a considerable period of latency. The length of such a period seems to be a measure of the advance of a master mind over his contemporaries. The latency period of chromatography lasted for 25 years and, during that time, only a few and scattered applications were reported. Tswett's detailed monograph appeared in 1910, in Russian, and, in the subsequent few years, Dhéré and Vegezzi (1916) made use of it. In the United States, Palmer (1922) was certainly one of the first investigators who carried out extensive chromatographic studies, and it is evident from his remarkable monograph on carotenoids that he realized the importance of the method. Although Tswett had demonstrated the duality of chlorophyll, it is a historically interesting fact that the great pioneer in that field, Willstätter, so far as is known, never employed chromatography.

The latency period of chromatography was broken in 1931, by Kuhn and Lederer as well as by Kuhn, Winterstein, and Lederer (1931) who, working on a preparative scale, resolved plant carotene into several components, and fulfilled Tswett's prophecy of 21 years before, that

"very likely carotene is not a chemical entity but a mixture of two or more homologues which it may be possible to separate from each other by means of adsorption methods . . ."

Fifteen years have elapsed since the resolution of carotene, and still chromatography is essentially empirical in character. The mathematical analysis of its procedures, as carried out by competent authors, so far has had scarcely any influence on practical experimentation.

One of the factors which, at present, hinder a more rapid theoretical development is the lack of a great number of commercially available, cheap adsorbents, expertly packed, promptly delivered, and of constant and guaranteed quality. By quality, of course, I do not mean a grade or a sieve number on the label, but rather the statement of the result of a careful standardization, based, for example, on the behavior of the material in the presence of well-selected dyes, as suggested by Brockmann and Schodder (1941). In spite of the increasing importance of chromatography in some commercial processes, industrial circles have so far shown very little interest in this problem. The present deplorable state of affairs reminds us of that in Bunsen's laboratory a century ago, when each scientific worker first had to make and calibrate his burets and pipets before he could begin to work.

## SCOPE

In spite of many hindrances, more than a thousand papers have appeared, during the last decade, in which successful chromatographic experiments are reported. These many data make it possible to evaluate the scope of the method. For more detailed information, recent monographs, with bibliographies, by the following authors may be consulted: Brockmann (1943); Cook (1941); Hesse (1943); Strain (1945); Vetter (1939); Williams (1946); Willstaedt (1939); Zechmeister and Cholnoky (1943).

Although chromatography seemed, earlier, to be of special advantage to investigators of naturally occurring substances, we are now witnessing its introduction into syntheses. The purification and check of purity of artifacts by chromatographic filtration will, for example, probably soon become a routine operation. Its evident advantage over other procedures is that pigmented or fluorescing by-products and contaminants may easily be located on the column and identified if necessary. Such a process also offers an efficient analytical check for the standardization of industrial products.

Other well-known applications of chromatography can be placed under the headings: resolution of a mixture, accumulation of substances which occur in great dilution, and checking of the homogeneity of a compound as well as its identity or non-identity with another sample by a mixed chromatogram test.

**Location of Zones.** Since the majority of carbon compounds are colorless, a rapid and reliable method for the location of invisible zones is often required. Under favorable conditions, no special procedures may be necessary for this purpose. For example, Trappe (1941) reports that silicic acid, which appears to be translucent when moistened with a pure solvent, loses this property at the section where a zone is present.

The inspection of the column with a portable ultraviolet lamp during the chromatographic process, either in a dark room or under a black cloth, is becoming increasingly popular (Karrer and Schöpp, 1934; Winterstein and Schön, 1934). On the other hand, strongly fluorescent columns allow the location of numerous compounds which are able to quench the fluorescence of the adsorbent and appear as dark zones (Sease, 1947; Brockmann and Volpers, 1947). Claesson (1947) suggests the use of total reflection on a thick glass plate for the detection of invisible zone boundaries.

Insufficient use has been made, so far, of the brush method, *i.e.*, streaking the column with a brush which has been dipped into a suitable reagent (Zechmeister, Cholnoky, and Ujhelyi, 1936). Of course, such streaks can also be inspected in ultraviolet light. Considering the immense number of well-defined color reactions, there is almost no limit to their chromatographic application. For many unsaturated compounds, permanganate may be used, as has been shown, *e.g.*, for stilbene (Zechmeister and McNeely, 1942), and, especially, for a number of sugar derivatives and free carbohydrates by Wolfrom and his collaborators (Lew, Wolfrom, and Goepp, 1945, 1946; McNeely, Binkley, and Wolfrom, 1945; Binkley and Wolfrom, 1946; Georges, Bower, and Wolfrom, 1946).

The applications of color reactions may be carried out in other ways. Reagent paper strips can be attached to the lower end of the chromatographic tube for this purpose. The appearance of a positive reaction then indicates that a zone is just entering the filtrate. One can also test for the location of some compounds by applying a reagent to samples from different sections of the extruded column.

Determination of the position of zones containing biologically active substances may be made by means of special tests of various descriptions. Haller, Acree, and Potts (1944) prepared extracts of the abdominal tips of the virgin female gypsy moth and, after chromatography, located that section which contains the sex attractant by exposing evaporated eluates of empirically cut fractions to male moths. One fraction attracted 114 males, the other only 26.

**Sequence of Zones.** After having discussed the location of zones, we must necessarily consider their chromatographic sequence. The sequence of two or more given compounds cannot usually be predicted simply on the basis of the individual adsorption isotherms. As was pointed out, for example, for fatty acids (Cassidy, 1940) and for amino acids (Lottermoser and Edelmann, 1938; Schaaf and Reinhard, 1944), the mutual influence of the components of a mixture may constitute an overwhelming factor in determining their sequence. In some cases, predictions seem to be possible on the basis of the permanent dipoles. Arnold (1939) stated that the sequence of decreasing dipoles of *p*-, *m*-, and *o*-nitrophenol is also the sequence of decreasing adsorbabilities. However, no generalization seems to be possible on this basis.

In each case, the absolute and relative adsorbability of a compound will, of course, be a function of the molecular structure, although not every structural detail will be effective in determining the behavior on the active surface. The location of those groups which are responsible for the adsorption behavior in a given system will be of great importance for future theoretical research. This is a problem similar to that of the determination of haptene groups which are responsible for the fixation of a drug on an adsorbing tissue. Within a complicated molecule, several atomic groups may compete for the surface, and it seems that various orientations of the molecule in different chromatographic systems are possible.

If all compounds which are on the same column possess analogous structures (for example, if they are homologues or close analogues), then a change in the adsorbent and developer probably will affect each component in the same direction, and their relative sequence will remain unaltered in the new system.

However, if different functional groups or other structural features are present in the molecules of the compounds to be chromatographed, then the individual responses to a change of the system may be unequal. The adsorption affinity of one compound may be increased and that of another may be decreased, and these changes may even, under favorable circumstances, cause an inversion of the original sequence on the column.

The factors which influence the chromatographic sequence were discussed by Strain (1946) in a new and important communication (*cf.* also Strain, 1942).

Among many other observations, Strain, Manning, and Hardin (1944) found that, when petroleum ether was used as a developer, the sequence from top to bottom of the two chlorophylls and a carotenoid-alcohol, fucoxanthin, on sugar was:

chlorophyll *b*
chlorophyll *a*
fucoxanthin *a*.

In contrast, the addition of as little as 0.5 per cent propyl alcohol to the petroleum ether produced the following sequence:

fucoxanthin *a*
chlorophyll *b*
chlorophyll *a*.

Evidently, the two chlorophylls responded similarly to the change in the system and, hence, their relative sequence remained unchanged. Fucoxanthin, however, which is structurally very different from the chlorophylls, responded differently and moved to the top position in the new system.

As pointed out by Strain *et al.*, if an inversion can be produced simply by changing the ratio of two solvents, then there must exist a mixture which will cause no separation at all.

Another characteristic example of an inversion in which not the developer (benzene) but the adsorbent was changed was given by LeRosen (1942). It refers to the pair, lycopene, $C_{40}H_{56}$, which contains 13 double bonds (all in an open chain) but no hydroxyl, and cryptoxanthin, $C_{40}H_{56}O$, which contains 11 conjugated double bonds (two in rings) and also a hydroxyl group. On alumina or calcium carbonate, the sequence is:

cryptoxanthin
lycopene,

which shows that the presence of hydroxyl is responsible for a strong fixation, and that this effect overrules the presence of a greater number of double bonds in the hydrocarbon. In contrast, the sequence on calcium hydroxide is:

lycopene
cryptoxanthin.

In aqueous systems, a change in pH may cause inversion in a chromatographic sequence.

**Interaction between Column and Substance.** If we wish to delineate the scope of chromatography, we should also consider how far the adsorption process itself may cause chemical changes. It is common knowledge that, in thousands of experiments, changes did not occur or, to be more exact, that the starting material, after elution, could be recovered quantitatively. On the other hand, to deny in principle the possibility of conversions on the column would be equivalent to the statement that the surface of a powdered solid is unable to react with a solute.

In fact, some interesting chemical changes have been reported. They belong to two different types.

Chemical alterations of the substance may be caused not by an interaction proper between adsorbent and solute, but by the sensitivity of the compound in the adsorbed state toward oxygen, for example. Thus, some unsaturated fatty acids or amino acids undergo autoxidation on alumina or charcoal. This unwelcome process may be checked by degassing the adsorbent and then excluding air during all operations, or, most simply, in some cases, by a pre-treatment of the column material with an agent which counteracts the tendency toward oxidation.

A treatment with potassium cyanide or hydrogen cyanide will prevent catalytic autoxidations which are caused by traces of some heavy metals

(Tiselius, 1941; *cf.*, for example, Turba, Richter, and Kuchar, 1943; Schramm and Primosigh, 1943). It may also happen that such oxidations are promoted by light. Levy and Campbell (1939) observed, when chromatographing 2,3-benzanthracene, that a zone became orange on the side facing the window because of local photo-oxidation to a quinoid compound.

Concerning the chemical interactions proper between substance and column, the available experimental data can be grouped into two subclasses, although the border lines are far from well-defined. In a number of cases, we do not know exactly what happens in the column, and are unable to give definite chemical equations. It is well known, for example, that chlorophyll suffers an irreversible change on alumina, calcium carbonate, sodium sulfate, fuller's earth, but can be chromatographed satisfactorily on sugar or inulin (Winterstein and Stein, 1933; Mackinney, 1938). Vitamin $K_1$ is biologically inactivated by magnesia or alumina, but is stable on sugar (Dam and Lewis, 1937).

Of several well-defined reactions which occur on the column, the following may be cited. Dimerization is observed if acetone is filtered through activated alumina, the product being diacetone-alcohol (Hesse, Reicheneder, and Eysenbach, 1938). Polymerization and structural isomerization of a number of essential oils have been described by Carlsohn and Müller (1938). Such a catalytic effect of the frankonite column is increased by dehydration of the adsorbent, *in vacuo*, over phosphorous pentoxide. Possibly, other similar changes in labile structures are due simply to exceptionally high heats of adsorption.

Hydrolytic elimination of acyl groups may occur on alumina when triglycerides are chromatographed (Trappe, 1940). Cahn and Phipers (1937) reported that, on alkaline alumina, a hydrolysis of diacetyltoxicarol takes place, and that the reaction may be prevented by pre-treating the adsorbent with acetic acid or phenol. These treatments, however, diminish the adsorbing capacity.

An elimination of water or alcohol may occur on the column. When rhodin-$g_1$-trimethylester was developed with methanol on talc, acetal formation took place because of the acidity of the adsorbent. However, on neutral talc, no such conversion occurred (Fischer and Conrad, 1939). According to Brockmann and Junge (1943), alcohol was split off by alumina from some anthocyanidine-like synthetic pigments, a reaction which also takes place when the solution is refluxed instead of being chromatographed.

Column reactions are demonstrated conspicuously when color is produced by the adsorption of a colorless substance, or when the color of the starting material is fundamentally altered. Sometimes, color is evident in the presence of a non-polar developer but disappears on subsequent application of a polar solvent. Triphenylcarbinol is adsorbed by alumina from its colorless benzene or chloroform solution with yellow color and by silica gel with brownish-yellow color (Weitz and Schmidt, 1939).

A well-known reaction, detected by Takahashi and Kawakami (1923), takes place when polyene pigments such as carotenoids, faintly colored polyenes such as diphenyloctatetraene, or colorless polyenes such as vitamin A or phytofluene, are adsorbed from petroleum ether or benzene on acid earths, *e.g.,* filtrols. A dark blue or greenish-blue zone appears immediately at the top of the column, but disappears upon the elution with alcohol or acetone. The formation of color may be irreversible, in the sense that after elution the original extinction curve, for example, of phytofluene no longer is evident (Zechmeister and Sandoval, 1945). The formation of color on acid earths was explained recently by Meunier (1942) as a donation of unshared electrons by the compound to those atoms of the adsorbent which had possessed incomplete octets. The molecule thus suffers far-reaching polarization on the adsorbent and forms strongly resonating structures.

The appearance of an orange pigment on calcium hydroxide or alumina columns during the chromatography of vitamin A has been observed in several laboratories (*cf.* Castle, Gillam, Heilbron, and Thompson, 1934). In this connection, Holmes and Corbet (1939) warned against "the blind use of adsorption columns". According to Meunier and Vinet (1945), the formation of the orange pigment is the result of the elimination of one mole of water from two molecules of the vitamin with the formation of a dipolyene ether.

The possibility of such complications should not discourage the chromatographer, since an unwelcome disturbance in experimentation may soon become a new and useful tool. In fact, some changes which occur on the column can be conveniently used for preparative purposes, as, *e.g.,* for freeing a base from its salt, for conversion of one salt into another, for splitting of addition compounds, etc.

Thus, some curare alkaloids, which were available to Wieland and Pistor (1938) in the form of their anthraquinone sulfonates, were filtered through HCl-pretreated alumina. The anthraquinone sulfonic acid was retained on the column and was replaced by hydrochloric acid in the solution, whereupon the hydrochloride of the alkaloid could be crystallized from the chromatographic filtrate. However, on basic alumina, the free alkaloid was obtained. According to Plattner and Pfau (1937) as well as other investigators, the cleanest method for the liberation of many terpenes, sterols, etc., from their picrates or trinitrobenzoates is a filtration through alumina. The nitro derivative forms a colored zone, while the freed colorless component passes into the filtrate. Similarly, as was reported by Weygand and Birkofer (1939), the prosthetic group of "old" yellow enzyme is retained on frankonite, but the protein component is washed into the filtrate.

As far as I know, no use has yet been made of the following suggestion of Tswett: "For special aims . . . . one will purposely use chemically effective adsorbents (hydrolyzers, reducers, oxidizers)."

May I mention that chromatography can also influence the course of organic chemical work in many indirect ways. A rather interesting case was reported by Kuhn and Strőbele (1937), whose crude 2-nitro-4, 5-dimethylaniline sample, which easily formed glucosides, lost this ability upon chromatographic purification, as it turned out, because a specific catalyst was eliminated by the column. This catalyst was ammonium chloride, the addition of which restored the ease of the glucoside synthesis.

## METHODS

Although the title of this survey includes also the methods of chromatography, only a short outline can be given. Reference must be made to other contributions to the present Conference.

It seems that, at the present time, there are five methods which promise further important developments:

(1) A characteristic feature of recent experimental work is the further perfection of the liquid chromatogram procedure, *i.e.*, the fractional washing of individual compounds into the filtrate. Especially, in the field of di- and tri-terpenes or steroids, including sex hormones, such methods have been worked out in the laboratories of Reichstein and of Ruzicka with great success. They are reported in a great number of papers which appeared in the *Helvetica Chimica Acta* during recent years. In some cases, more than a dozen solvents or solvent mixtures with increasing developing and eluting power were applied successively and, thus, permitted the use of a relatively small column for a large-scale experiment. The very great solvent requirement is an inconvenience but, on the other hand, the fractionation is satisfactory even if the individual compounds are not present in distinct zones but are washed out one by one from a mixed adsorbate. So far as the author knows, no general survey is available in this field.

(2) A further, promising direction of research is the use of exchangers of the zeolite type as column materials. The practical difference in the function of an adsorbent proper and an exchange adsorbent is that, in the latter instance, the process necessarily involves a release of some constituent from the solid surface to the solvent. In most cases, aqueous or water-containing media have been used, and the process can be characterized as an ion exchange. A new stimulus to exchange chromatography has been given by the study of synthetic resins, the selective exchange properties of which were discovered by Adams and Holmes (1935). These procedures are now being used extensively, for example, in the separation of amino acids of different types. The method has

much in common with ordinary chromatography and, thus, one may discuss the observed phenomena in terms of exchange-affinity, exchange-displacement, exchange-chromatographic sequence, and so forth. After adequate conditioning, either cation or anion exchange can be carried out. I may refer to the review by Myers (1942).

(3) Another important experimental technique, *Partition Chromatography,* was introduced with success by Martin and Synge (1941; *cf.* also Gordon, Martin, and Synge 1943, 1944, and connected papers). In this technique, the solid column is essentially a support for a static liquid phase, while a mobile liquid phase flows through the adsorbent. In the usual form of chromatography, a very great number of adsorptions and elutions is responsible for the resolution of a mixture, but in partition chromatography a very great number of consecutive partitions of the substance occur between the two liquid phases. Thus, differences in individual partition coefficients play a decisive role in the success of the experiment. The authors mentioned worked with silica gel and separated acetamino acids. However, it was shown by Synge (1944) that, on starch, free amino acids can be handled as well. A competent survey of this whole field has been given by Martin and Synge (1945).

(4) In connection with the investigations just mentioned, the use of paper strips for the resolution of mixtures as initiated by Schoenbein and by Goppelsroeder and surveyed by Reinholdt (1925), has assumed new shape and significance. Recently, Consden, Gordon, and Martin (1944) described their "two-dimensional chromatography" which is based on the partition principle. The liquid phases are, for example, water (in the cellulose) and a mobile developer. A droplet containing a very small amount of wool hydrolysate is placed on the corner of a quadrangular piece of paper and developed first with collidine along one side of the paper and then with a dilute phenol solution in a direction perpendicular to the first. The position of the resulting amino acid spots can be demonstrated by spraying with ninhydrin solution and gentle heating.

(5) Finally, mention should be made of the chromatographic boundary method invented by Tiselius (1940). A solution is forced through an adsorbent under conditions which make possible a continuous optical characterization of the emerging liquid by means of a device which records the refractive indices (Claesson, 1944). The individual compounds have characteristic retention volumes and show a sudden breakthrough. This method is acquiring considerable analytical importance in many fields of organic chemistry and biochemistry. It can be carried out as a "frontal analysis" (without developer), as an "elution analysis" (developed with a solvent), or as a "displacement development" (using a strongly adsorbed displacer). These methods have been reviewed by Tiselius (1942) and by Claesson (1946).

When one surveys all the methods and applications of chromatography which are available at the present time, including those for the separation of isotopes, for inorganic analysis, and for technological research, one receives the impression of an extended and manifold picture. This vista contrasts sharply with that which Tswett was able to see during the fourteen years between the publication of his first article on chromatography and his death. He enjoyed neither personal happiness nor proper working conditions, and he received scarcely any acknowledgment during his lifetime. Furthermore, his life was made difficult and, finally, destroyed by the events of the first World War and its consequences. Nevertheless, one has the definite impression, in reading his papers, that he believed with almost fanatical faith in his pioneer ideas.

The present short survey is dedicated to the memory of Michael Tswett.

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# CHROMATOGRAPHY: A PROBLEM IN KINETICS

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The theory of chromatography has been discussed by several writers.[1–9] For the most part, the problem has been treated from the point of view of instantaneous equilibrium between solution and adsorbent. Walter[8] has, however, given a kinetic treatment of the problem for the case of ion exchange between solution and solid. The complete solution of this latter problem has been given by the present writer.[10] The present paper, after a review of pertinent parts of the equilibrium theory, will develop mathematical descriptions of single solute chromatography for the two simplest reasonable mechanisms of adsorption considered as a kinetic process. The results of some experiments in which rate data were obtained are reported, and are discussed from the point of view of the kinetic theory. The multiple solute problem is mentioned.

## THEORETICAL PART

**The Equations of Conservation.** The equations of conservation for flow processes such as that of chromatography are well known, but are restated here in the interest of obtaining a connected account of the theory. We consider that the experiment is carried out in a uniform tube uniformly packed with adsorbent. Let the distance from the input end of the tube be measured in terms of the mass of the adsorbent contained therein, say $x$ (grams). We will consider only cases for constant input rate of solution or solvent and will denote this rate by $\dot{V}$ (milliliters per minute). Let time be denoted by $t$ (minutes); concentration, by $c$ (millimoles per milliliter); "concentration" of adsorbed material, by $q$ (millimoles per gram); and the free space or pore volume of the adsorbent, by $m$

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[1] **Wilson, J. N. J. Am. Chem. Soc. 62: 1583. 1940.**
[2] **Martin, A. J. P., & R. L. M. Synge.** Biochem. J. **35: 1358. 1941.**
[3] **DeVault, D. J. Am. Chem. Soc. 65: 532. 1943.**
[4] **Weiss, J. J. Chem. Soc. 145: 297. 1943.**
[5] **Offord, A. C., & J. Weiss.** Nature **155: 725. 1945.**
[6] **Glückauf, E.** Ibid. **156: 205. 1945.**
[7] **Walter, J. E. J. Chem. Phys. 13: 229. 1945.**
[8] **Walter, J. E.** Ibid. **13: 332. 1945.**
[9] **Glückauf, E.** Proc. Roy. Soc. London **186: (1004): 35. 1946.**
[10] **Thomas, H. C. J. Am. Chem. Soc. 66: 1664. 1944.**

(milliliters per gram). The conservation condition demands that the solute entering a layer $dx$ of the tube in time $\delta t$ be accounted for as follows:

$$c\dot{V}\,\delta t = \left(c + \frac{\partial c}{\partial x}\,dx\right)\dot{V}\,\delta t + m\,dx\,\frac{\partial c}{\partial t}\,\delta t + dx\,\frac{\partial q}{\partial t}\,\delta t;$$

whence

$$\frac{\partial c}{\partial x} + \frac{m}{\dot{V}}\frac{\partial c}{\partial t} + \frac{1}{\dot{V}}\frac{\partial q}{\partial t} = 0, \qquad (1)$$

or, for constant flow rate,

$$\frac{\partial c}{\partial x} + m\frac{\partial c}{\partial V} + \frac{\partial q}{\partial V} = 0. \qquad (1a)$$

In the formulation of the kinetic problem, it will be convenient to use the equation in which $\dot{V}$ appears explicitly; for the equilibrium theory, the equation in $V$ and $x$ is, of course, adequate.

For most purposes, it will be convenient to change to independent variables more natural to the problem, namely:

$$\left.\begin{aligned} x &= x, && \text{the mass of adsorbent upstream}\\ y &= \dot{V}t - mx &&\\ &= V - mx, && \text{the volume of solution downstream}\end{aligned}\right\} \qquad (2)$$

In these terms, EQUATION 1 becomes

$$\frac{\partial c}{\partial x} + \frac{\partial q}{\partial y} = 0. \qquad (3)$$

The integral form of the conservation condition (EQUATION 3) may be written down at once. Insofar as $c$ and $q$ are piecewise differentiable functions of $x$ and $y$, EQUATION 3 ensures the existence of the line integral, independent of path,

$$F = \int_{x_1,\,y_1}^{x_2,\,y_2} (q\,dx - c\,dy). \qquad (4)$$

If the path of integration can be taken from $x = 0$ to some point at and beyond which $c$ and $q$ vanish, then $F$ is the total amount of solute, adsorbed and in solution, in the column.

**The Equilibrium Theory for Single Solutes.** Most of the previous discussions of the theory of chromatography have started with EQUATIONS 1 or 3 and have introduced the assumption of instantaneous equilibrium between solution and adsorbent. It has, of course, been realized that, at best, this assumption is an approximation. The idea is formulated by the introduction of the adsorption isotherm,

$$q = f(c). \tag{5}$$

We are here measuring $f$ per gram of adsorbent. EQUATION 3 now becomes

$$\frac{\partial c}{\partial x} + f'(c)\,\frac{\partial c}{\partial y} = 0. \tag{6}$$

Wilson[1] has discussed the solutions of this equation given by $c =$ constant. The method of characteristics[11] enables one to obtain other solutions of the equation. The equations of an integral surface of EQUATION 6, described by the parameter, $s$, passing through a given initial distribution of $c$, say $c_i\,(\tau)$, are

$$\left.\begin{array}{l} x = s + x_i\,(\tau) \\ y = f'(c)\;s + y_i\;(\tau) \\ c = c_i\,(\tau) \end{array}\right\}. \tag{7}$$

in which $\tau$ is the variable which maps the initial distribution. These results are subject to the condition that the initial ($s = 0$) distribution of $c$ is continuous and continuously differentiable. As has been shown by DeVault[3] and by Weiss,[4] this solution will eventually give rise to a physically absurd situation corresponding to a many-valued concentration at a point in the tube. This situation has been taken as an indication of the formation of a sharp front in the chromatogram. The difficulty is removed by replacing it with a discontinuity in the concentration at such a point as will give conservation of matter in the tube. For the saturation of an initially empty tube with solution of concentration $c_0$, the discontinuity is thus placed at the position $x_D$ given by

$$\left.\begin{array}{c} Vc_0 = \left\{mc_0 + f(c_0)\right\}\,x_D \\ yc_0 = x_D\,q_0. \end{array}\right\} \tag{8}$$

In the discussion of the development or elution process from this point of view, it is noted that, according to the differential equation, the rate of movement of a point of given concentration in the chromatogram is given by

$$\left.\begin{array}{l} \left(\dfrac{dx}{dV}\right)_c = \dfrac{1}{m + f'(c)} \\ \text{or} \\ \left(\dfrac{dx}{dy}\right)_c = \dfrac{1}{f'(c)}\,. \end{array}\right\} \tag{9}$$

Thus, for an isotherm convex upward, *i.e.*, such that $f'\,(c_1) > f'\,(c_2)$ for $c_1 < c_2$, the points of lower concentration move more slowly. Accordinging to this idea, a diffuse trailing boundary is formed on development of an initially sharp chromatogram. It should be noted that we are here retaining the solution of the differential equation for the description of the trailing boundary but are discarding it for the leading boundary of the same chromatogram. This procedure may be mathematically arbitrary, but it is physically possible: the same assignment of position of EQUATION 8 as was used in the saturation case will give conservation of matter in the case of elution with pure solvent.

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[11] **Courant, R., & D. Hilbert.** Methoden der Mathematischen Physik **II:** 51–55. Julius Springer. Berlin. 1937.

For comparison, later, with experimental and further theoretical results, we shall write the expression for an equilibrium trailing boundary based on the Langmuir isotherm

$$q = \frac{Kac}{1 + Kc}, \tag{10}$$

namely

$$Kax = (Kc + 1)^2 y. \tag{11}$$

This equation has been given implicitly by DeVault[3] and explicitly by Weiss.[4] In EQUATION 10, the constant is taken so that large $K$ means strong adsorption. The total adsorption capacity is denoted by $a$. For a chromatogram eluted from a tube initially saturated at concentration $c_0$,[12] EQUATION 11 states that the effluent concentration will vanish at a volume given by

$$y = Kax. \tag{12}$$

For such a chromatogram, the theory predicts that the effluent concentration will have the value $c_0$ until $y$ attains the value

$$y_1 = \frac{Kax}{(Kc_0 + 1)^2}, \tag{13}$$

when the concentration will abruptly begin to decrease.

The equilibrium theory of chromatography has the advantage of simplicity and is, hence, of considerable use in the qualitative interpretation of experiments. Quantitatively it has been little tested. It cannot, of course, be expected to apply in the many cases where it is easy to show that equilibrium is not attained. We can hope to gain some insight into the detailed structure of a chromatogram if we put into the theory some reasonable mechanism which will give a lag in the attainment of equilibrium.

**A Kinetic Theory of Chromatography.** The most obvious way to introduce the time factor into the theory is to take explicit account of the finite rate of adsorption. Let us suppose that the rate is determined by chemical effects, *i.e.*, that diffusion is not a rate-determining factor. Perhaps the simplest way in which this idea can be formulated is by the assumption of a reaction law, not unreasonable in itself, which will lead directly to a Langmuir type isotherm at equilibrium. In this connection, it should be noted that there is no unique such mechanism[13]; a Langmuir type isotherm experimentally found is no guarantee of simple kinetics. We will suppose that the rate of desorption is first order with respect to the concentration of adsorbed material, and that the rate of adsorption is proportional to the product of the "concentration of empty holes" on the adsorbent and the concentration in solution of the material being adsorbed. Thus, we write

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[12] Holmes, H. N., H. Cassidy, R. S. Manly, & E. R. Hartzler. J. Am. Chem. Soc. 57: 1990. 1935.

$$\left(\frac{\partial q}{\partial t}\right)_x = \dot{V}\left(\frac{\partial q}{\partial y}\right)_x = k_1(a - q)c - k_2 q, \tag{14}$$

in which $k_1$ (ml. mmol.$^{-1}$ min.$^{-1}$) and $k_2$ (min.$^{-1}$) are the velocity constants in terms of which we hope to describe the process. The resulting equilibrium relation is, of course,

$$q = \frac{k_1 ac}{k_2 + k_1 c} = \frac{Kac}{1 + Kc}, \tag{15}$$

with

$$K = {}^{k_1}\!/_{k_2}.$$

For very weak adsorbers, or for the early stages of any such adsorption process, EQUATION 14 may be simplified to read

$$\dot{V}\left(\frac{\partial q}{\partial y}\right)_x = k_1 ac - k_2 q, \tag{16}$$

giving the linear isotherm

$$q = Kac. \tag{17}$$

EQUATIONS 16 and 17 are written down explicitly because it will be simpler to develop *ab initio* for this case the kinetic theory of the corresponding chromatographic process. In what follows, the mechanism represented by EQUATION 14 will be referred to as *Langmuir kinetics;* the simpler case given by EQUATION 16 will be termed *linear kinetics.* The formulae resulting from linear kinetics are appreciably simpler than those for the more general case. They will be useful in some applications and will serve as a guide in the application of the more complex formulae.

As we have seen, the conservation equation (3) ensures the existence of a function, $F$, such that

$$dF = q\,dx - c\,dy, \tag{18}$$

with, then,

$$\left.\begin{aligned} c &= -\frac{\partial F}{\partial y} \\ q &= \frac{\partial F}{\partial x} \end{aligned}\right\} \tag{19}$$

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[13] **Fowler, R. H., & E. A. Guggenheim.** Statistical Thermodynamics: 427. Cambridge University Press. 1939.

The corresponding differential equations for the chromatographic processes may at once be written down. We have for the linear case

$$\frac{\partial^2 F}{\partial x \partial y} + A\frac{\partial F}{\partial x} + B\frac{\partial F}{\partial y} = 0, \tag{20}$$

and for the Langmuir case

$$\frac{\partial^2 F}{\partial x \partial y} + A\frac{\partial F}{\partial x} + B\frac{\partial F}{\partial y} + C\frac{\partial F}{\partial x}\,\frac{\partial F}{\partial y} = 0, \tag{21}$$

in which we have written for convenience

$$A = \frac{k_2}{\dot{V}}, \quad B = \frac{k_1 a}{\dot{V}}, \quad C = -\frac{k_1}{\dot{V}} \tag{22}$$

Since we will need them in the application of EQUATION 21, we state here the definitions of two additional constants,

$$\alpha = \frac{k_1 c_0 + k_2}{\dot{V}}; \; \beta = \frac{1}{\dot{V}}\;\frac{k_1\; k_2\; a}{k_1\; c_0 + k_2}, \tag{22a}$$

such that $AB = \alpha\beta$.

By appropriate substitutions, EQUATIONS 20 and 21 may be reduced to a single form. If we put, in EQUATION 20,

$$F = e^{-(Bx + Ay)}\;\Psi\;(x, y), \tag{23}$$

there results

$$\frac{\partial^2\,\Psi}{\partial x \partial y} = AB\,\Psi. \tag{24}$$

If, in EQUATION 21, we put[10]

$$CF = \ln\left\{e^{-(Bx + Ay)}\;\Phi\;(x,\,y)\right\} \tag{25}$$

we again get

$$\frac{\partial^2\,\Phi}{\partial x \partial y} = AB\,\Phi. \tag{26}$$

In the case of the linear equation (20), the expressions (19) give us the following formulae for the calculation of the concentrations:

$$\left.\begin{aligned} c &= e^{-(Bx + Ay)}\left\{A\,\Psi - \frac{\partial\Psi}{\partial y}\right\} \\ \text{and} \qquad q &= -\,e^{-(Bx + Ay)}\left\{B\,\Psi - \frac{\partial\Psi}{\partial x}\right\}\end{aligned}\right\} \tag{27}$$

The corresponding formulae for the Langmuir kinetics are

$$\left.\begin{aligned} c &= \frac{1}{C}\left\{A - \frac{\partial \ln \Phi}{\partial y}\right\} \\ \text{and} \qquad q &= -\frac{1}{C}\left\{B - \frac{\partial \ln \Phi}{\partial x}\right\} \end{aligned}\right\} \tag{28}$$

**The Boundary Value Problems for Initially Empty and Initially Saturated Columns.** Solutions of the differential EQUATIONS 24 and 26 have now to be obtained which reduce to specified initial conditions in the chromatographic column. In this paper, solutions will be obtained for initially empty and initially saturated columns. Thus, we obtain formulae applicable to the effluent from the columns. Experimentally, at least from the point of view of a test of the theory, these are the most interesting cases. Only for such effluents have accurate data been obtained. In the future, it may become of interest to examine quantitatively the structure of the bands within the tube. The corresponding mathematical problem, which has not been carried through, promises to be more complex than the one treated here.

The boundary values of the functions $\Psi$ and $\Phi$ are specified as follows.

SATURATION OF COLUMN INITIALLY EMPTY.

$$\left.\begin{aligned} x = 0, \quad & y \geq 0, \quad c = c_0, \\ x \geq 0, \quad & y = 0, \quad q = 0. \end{aligned}\right\} \tag{29}$$

*Linear kinetics.* The corresponding boundary values of $\Psi$ follow from EQUATIONS 27. Using the condition on the concentration in solution at the input of the column, we find

$$\frac{d\,\Psi(0,y)}{dy} - A\,\Psi(0,y) + c_0\,e^{Ay} = 0,$$

the solution of which may readily be verified to be

$$\Psi(0,y) = (1 - c_0\,y)\,e^{Ay}. \tag{30}$$

From the equation determining $q$, we have

$$\frac{d\,\Psi(x,0)}{dx} - B\,\Psi(x,0) = 0,$$

whence

$$\Psi(x,0) = e^{Bx}. \tag{30a}$$

In the deduction of these the expressions $\Psi$ (0,0) has been put equal to unity, so that $c = c_0$ for $x = 0$, $y = 0$.

Even more simply, in the more general case, we find from EQUATIONS 28 and 29,

$$\Phi\,(0, y) = e^{\alpha y} \tag{31}$$

$$\Phi\,(x, 0) = e^{Bx} \tag{31a}$$

The quantity $\alpha$ is defined in EQUATION 22a.

ELUTION OF COLUMN INITIALLY SATURATED. The boundary values of the functions for the case of the elution by pure solvent of a column initially saturated at $c = c_0$ are specified by

$$\left.\begin{array}{lll} x = 0, & y \geq 0, & c = 0 \\ x \geq 0, & y = 0, & q = q_0 \end{array}\right\} \tag{32}$$

The value of $q_0$ is given by the isotherm (EQUATIONS 15 or 17) for $c = c_0$. The corresponding sets of values of $\Psi$ and $\Phi$ are

$$\Psi\,(0, y) = e^{Ay} \tag{33}$$

$$\Psi\,(x, 0) = (1 + q_0\,x)\,e^{Bx} \tag{33a}$$

and

$$\Phi\,(0, y) = e^{Ay} \tag{34}$$

$$\Phi\,(x, 0) = e^{Bx} \tag{34a}$$

Solutions of EQUATIONS 24 and 26 for the above sets of boundary conditions can be obtained by several means. The method of the Laplace transform leads to a contour integral which is most convenient as a starting point for the discussion of the solution. The classical method of Riemann leads directly to the unique[14] results which must be obtained. The simplest method is to take results which have been obtained for a similar problem[10] and transcribe them for the present case. These results may be summarized as follows. The equation

$$\frac{\partial^2 \varphi}{\partial u\,\partial v} = \varphi \tag{35}$$

is satisfied by the exponential $e^{u+v}$, by the Bessel function

$$I_0\,(2\sqrt{uv}) = \sum_{m=0}^{\infty} \frac{u^m\,v^m}{m!\,m!} \tag{36}$$

and by the definite integral

$$\varphi\,(u, v) = e^u \int_0^u e^{-t}\,I_0\,(2\,\sqrt{v\,t})\,dt = \sum_{0 \leq n < m}^{\infty} \frac{u^m\,v^n}{m!\,n!} \tag{37}$$

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[14] Courant, R., & D. Hilbert. *Op. cit.* II: 311–317.

Some of the important properties of the integral $\varphi(u,v)$ are repeated here for convenience:

$$\varphi(u,v) + \varphi(v,u) = e^{u+v} - I_0(2\sqrt{uv}) \tag{38}$$

$$\frac{\partial\varphi(u,v)}{\partial u} = \varphi(u,v) + I_0(2\sqrt{uv}) \tag{39}$$

$$\frac{\partial\varphi(u,v)}{\partial v} = \varphi(u,v) - \frac{\partial}{\partial v} I_0(2\sqrt{uv}) \tag{40}$$

$$\left.\begin{aligned} \varphi(0,v) &= 0 \\ \varphi(u,0) &= e^u - 1 \end{aligned}\right\} \tag{41}$$

and

$$\lim_{u\to\infty} e^{-(u+v)}\varphi(u,v) = 1. \tag{42}$$

Solutions of the linear EQUATION 35 can be constructed from linear combinations of $I_0(2\sqrt{uv})$ and various $\varphi$'s. The character of the solutions can be modified to fit different boundary conditions by applying to them a suitable operator which commutes with $\partial^2/\partial u\partial v$; the resulting functions remain solutions of the differential equation. Of the infinity of such operators, the most important for our purposes are

$$\frac{\partial}{\partial u},\quad \frac{\partial}{\partial v},\quad u\frac{\partial}{\partial u} - v\frac{\partial}{\partial v}. \tag{43}$$

For example, the boundary values (EQUATION 30) for the saturation of a column under linear kinetics suggest that we try as a solution

$$\begin{aligned} \Psi &= e^{Bx+Ay} - \frac{c_0}{A}\left(y\frac{\partial}{\partial y} - x\frac{\partial}{\partial x}\right)\varphi(Ay,Bx) \\ &= e^{Bx+Ay} - c_0\Big[\, y\left\{\varphi(Ay,Bx) + I_0(2\sqrt{ABxy})\right\} \\ &\quad - \frac{B}{A}\,x\left\{\varphi(Ay,Bx) - \frac{\partial}{\partial Bx}\, I_0(2\sqrt{ABxy})\right\}\Big] \end{aligned} \tag{44}$$

This expression fulfills the prescribed boundary conditions and is, therefore, the solution desired. The solution for the elution of a saturated tube (linear kinetics) is

$$\Psi = e^{Bx+Ay} + \frac{q_0}{B}\left(x\frac{\partial}{\partial x} - y\frac{\partial}{\partial z}\right)\varphi(Bx,Ay). \tag{45}$$

With Langmuir kinetics, the appropriate solutions are, for the column initially empty,

$$\Phi = I_0(2\sqrt{ABxy}) + \varphi(Bx,Ay) + \varphi(\alpha y,\beta x), \tag{46}$$

and, for the column initially saturated,

$$\Phi = I_0(2\sqrt{ABxy}) + \varphi(Ay,Bx) + \varphi(\beta x,\alpha y). \tag{47}$$

**The Concentrations.** With the aid of the differentiation formulae (Equations 39 and 40), the introduction of the solutions of the preceding paragraph into Equations 27 and 28 is a simple matter. The results are as follows:

### Linear Kinetics

*Saturation*

$$\frac{c}{c_0} = e^{-(Bx + Ay)} \left\{ I_0 (2 \sqrt{ABxy}) + \varphi (Ay, Bx) \right\} \tag{48}$$

$$\frac{q}{q_0} = e^{-(Bx + Ay)} \varphi (Ay, Bx). \tag{49}$$

*Elution*

$$\frac{c}{c_0} = e^{-(Bx + Ay)} \varphi (Bx, Ay) \tag{50}$$

$$\frac{q}{q_0} = e^{-(Bx + Ay)} \left\{ I_0 (2 \sqrt{ABxy}) + \varphi (Bx, Ay) \right\} \tag{51}$$

The functions involved being all positive quantities, it is immediately apparent how the concentration of the solution leads in the saturation case and lags in the elution case, as compared to the concentration of adsorbed material.

### Langmuir Kinetics

*Saturation*

$$\frac{c}{c_0} = \frac{I_0 (2 \sqrt{ABxy}) + \varphi (\alpha y, \beta x)}{I_0 (2 \sqrt{ABxy}) + \varphi (\alpha y, \beta x) + \varphi (Bx, Ay)} \tag{52}$$

$$\frac{q}{q_0} = \frac{\varphi (\alpha y, \beta x)}{I_0 (2 \sqrt{ABxy}) + \varphi (\alpha y, \beta x) + \varphi (Bx, Ay)} \tag{53}$$

*Elution*

$$\frac{c}{c_0} = \frac{\varphi (\beta x, \alpha y)}{I_0 (2 \sqrt{ABxy}) + \varphi (\beta x, \alpha y) + \varphi (Ay, Bx)} \tag{54}$$

$$\frac{q}{q_0} = \frac{I_0 (2 \sqrt{ABxy}) + \varphi (\beta x, \alpha y)}{I_0 (2 \sqrt{ABxy}) + \varphi (\beta x, \alpha y) + \varphi (Ay, Bx)} \tag{55}$$

In passing, we may note that neglecting $k_1 c_0$ as compared to $k_2$, *i.e.*, making $\alpha = A$, $\beta = B$, reduces Equations 52-55 to the corresponding formulae for the linear case.

It should be noted that the results for Langmuir kinetics are essentially more complex than those for the linear case. They involve, explicitly, the initial concentration of the solution with which the column is being or was saturated. Thus, our theory predicts that if and only if the kinetics are linear will the behavior of the chromatogram be independent of initial concentration. This independence will, then, always obtain with sufficiently dilute solutions.

It is of some interest to notice that, in the case of radial flow through a disc of adsorbent of uniform thickness, one obtains the conservation equation (3) in an identical form if the variables are defined as follows:

$x = \pi r^2 d$, total mass of adsorbent within a circle of radius $r$;

$y = \dot{V} t - m\pi r^2 d$, total volume through a circle of radius $r$.

Here, $m$ has the same meaning as before; $d$ is the mass of adsorbent per unit area of the disc; and $\dot{V}$ is the total radial volume rate of flow. Thus, at sufficiently large distances from a small central input hole, the chromatogram in a disc will behave in a fashion entirely analogous to a chromatogram in a column if $x$ and $y$ are properly interpreted. Near the input hole, it would be necessary to reexamine the solutions of the equation, since the boundary values are not specified for $x = 0$.

**Limiting Cases for Slow Flow.** In the descriptions of the chromatographic process just developed, the essential characteristic from an experimental point of view is the explicit dependence of the various concentrations on the volume rate of flow through the tube. For a given set of velocity constants, the adsorption process will approach more and more nearly to equilibrium with decrease in flow rate. This fact finds its expression in the behavior of the various functions for small values of $\dot{V}$. A glance at the definitions 22 and 22a shows that we must examine the asymptotic behavior of the functions for large values of the arguments. This investigation is made easy by the use of an asymptotic expansion of the integral $\varphi\ (u,v)$ due to Professor Lars Onsager.[15] This development runs as follows:

$$\begin{aligned}
\varphi(u,v) = {} & \frac{1}{2}\left\{1 - H\left(\sqrt{v} - \sqrt{u}\right)\right\} e^{u+v} - \frac{r}{1+r} I_0 \\
& + \frac{1}{2}\left(\frac{1-r}{1+r}\right)\Big[ g\left\{2I_0 - \left(I_{\frac{1}{2}} + I_{-\frac{1}{2}}\right)\right\} \\
& + g^2\left\{6I_0 - 4\left(I_{\frac{1}{2}} + I_{-\frac{1}{2}}\right) + 2I_1\right\} \\
& + g^3\left\{20I_0 - 15\left(I_{\frac{1}{2}} + I_{-\frac{1}{2}}\right) + 12I_1 - \left(I_{\frac{3}{2}} + I_{-\frac{3}{2}}\right)\right\} \\
& + g^4\left\{70I_0 - 56\left(I_{\frac{1}{2}} + I_{-\frac{1}{2}}\right) + 56I_1 - 8\left(I_{\frac{3}{2}} + I_{-\frac{3}{2}}\right) + 2I_2\right\}\Big] \\
& + \left(1 - r^2\right)R_5,
\end{aligned} \tag{56}$$

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[15] Onsager, Lars. To be published.

in which

$$r = (u/v)^{\frac{1}{4}}, \quad g = r\,/\,(1 + r)^2$$

$$I_p = I_p\,(2\sqrt{uv}),\, H(Z) = \frac{2}{\sqrt{\pi}} \int_0^Z e^{-t^2} dt.$$

$R_5$ is a small remainder term.
For those values of $u$ and $v$ sufficiently large so that the required accuracy may be obtained by the use of the leading term in the asymptotic expansions of the Bessel functions, the expansion of $\varphi$ reduces to its first two terms. If, in addition, $|\sqrt{v} - \sqrt{u}|$ is sufficiently large, the error integral may be replaced by the first term of its asymptotic expansion, and we have a much simplified formula for the estimation of the behavior of our various expressions for the concentrations. The results for the effluent of a column may be summarized as follows.

## LINEAR KINETICS

*Saturation*

(a) $Ay < Bx$; that is, $Vc_o < (mc_o + Kac_o)x$; the column is not completely saturated.

$$\frac{c}{c_o} \simeq \frac{1}{2\sqrt{\pi}}\, e^{-(\sqrt{Bx} - \sqrt{Ay})^2} \frac{\left(\dfrac{Bx}{Ay}\right)^{\frac{1}{4}}}{\sqrt{Bx} - \sqrt{Ay}} \tag{57}$$

Here, the concentration is determined by the exponential and is, thus, extremely small in the region considered.

(b) $Ay = Bx$; $Vc_o = (mc_o + Kac_o)x$; just sufficient solution has been introduced to saturate the column under equilibrium conditions. Here we make use of EQUATION 38 and find

$$\frac{c}{c_o} \simeq \frac{1}{2} + \frac{1}{4\sqrt{\pi}} \frac{\sqrt{V}}{(k_1 k_2\, axy)^{\frac{1}{4}}} \tag{58}$$

Thus, the concentration ratio differs from ½ by an amount which decreases with the square root of the flow rate. The theory predicts, then, that all saturation curves for moderate rates will cross, for a given column, in the neighborhood of $c/c_o = ½$.

(c) $Ay > Bx$; $Vc_o > (mc_o + Kac_o)x$, *i.e.*, the column is working beyond the "break-through" point.

$$\frac{c}{c_o} \simeq 1 - \frac{1}{2\sqrt{\pi}}\, e^{-(\sqrt{Ay} - \sqrt{Bx})^2} \frac{\left(\dfrac{Bx}{Ay}\right)^{\frac{1}{4}}}{\sqrt{Ay} - \sqrt{Bx}} \tag{59}$$

In this region, the concentration differs by very little from its initial value.

### *Elution*

For this case, we find similar expressions. If $Ay < Bx$, *i.e.*, insufficient solvent completely to elute the column, we again get EQUATION 59. For $Ay = Bx$, we find, analogous to EQUATION 58,

$$\frac{c}{c_0} \simeq \frac{1}{2} - \frac{1}{4\sqrt{\pi}} \frac{\sqrt{\dot{V}}}{(k_1 k_2 axy)^{\frac{1}{4}}} \tag{60}$$

All elution curves cross in the neighborhood of $c/c_0 = \frac{1}{2}$. At larger volumes, $Ay > Bx$, EQUATION 57 reappears and we have concentrations exponentially close to zero.

Thus, in the limit for slow flow, the theory for linear kinetics reduces to the equilibrium theory for a linear isotherm. It predicts a chromatogram with sharp front and sharp rear boundary.

## LANGMUIR KINETICS

The results in the case of the more complete kinetics are similar. The condition of break-through for the column is

$$\left.\begin{aligned} \alpha y &> \beta x \\ Ay &< Bx \end{aligned}\right\} \tag{61}$$

Under these conditions, we may use EQUATION 42 in our estimate of the functions in EQUATION 52 and find for

### *Saturation*

$$\frac{c}{c_0} \simeq \frac{1}{1 + e^{\frac{k_1}{\dot{V}}(q_0 x - c_0 y)}} \tag{62}$$

Thus, for sufficiently small $\dot{V}$, $c \simeq 0$ or $c \simeq c_0$, according as $c_0 y < q_0 x$ or $c_0 y > q_0 x$. The condition, $q_0 x = c_0 y$, *i.e.*, the condition that the column is just saturated in the equilibrium case, gives the position of the sudden exponential rise in concentration. This result is in agreement with the assignment made necessary in the equilibrium theory by the appearance of the multiple valued concentrations in the leading boundary, EQUATION 8.

In the case of the elution of a saturated column, the limiting agreement of the two theories is even more striking. In the case of the equilibrium theory, the equation for the elution curve may easily be put in the form

$$\frac{c}{c_0} = \sqrt{\frac{y_1}{y}} \frac{\sqrt{Kax} - \sqrt{y}}{\sqrt{Kax} - \sqrt{y_1}} \tag{63}$$

in which $y_1$ is the value of the effluent volume at which the trailing boundary of the chromatogram appears (EQUATION 13). Inspection of the limiting form of EQUATION 54 for the conditions

$$\alpha y > \beta x$$
$$Ay < Bx$$

shows that the corresponding point is given for $\alpha y \rightarrow \beta x$, $y \rightarrow y_1$, which in view of the definitions of $\alpha$ and $\beta$ determines the same $y_1$. After some algebraic jugglery, the limiting form of EQUATION 54 may be put into just the form of EQUATION 63. Thus, again, we have complete agreement in the limit between the two theories. It should be pointed out, however, that for finite flow rate the kinetic theory gives a continuous decrease in concentration from $c_0$ and an exponential approach to zero at the far end of the tail of the chromatogram. In this respect, it more nearly represents the facts than does the equilibrium theory.

**The Determination of the Velocity Constants.** In the case of linear kinetics, fairly simple formulae may be obtained for the approximate determination of the velocity constants. Using this simplified picture, we cannot determine $k_1$ alone; it occurs only in the combination $k_1a$. The position of the mid-point of the saturation curve serves as approximate determination of $Ka$, for, by EQUATION 58, this point differs but little from that given by $y = Kax$. An estimate of the magnitude of $k_2$ may be had from the slope of the saturation curve at $c/c_0 = 1/2$. Differentiation of EQUATION 48 with respect to $y$ gives

$$\frac{\partial\,(c/c_0)}{\partial y} = e^{-(Bx + Ay)} \frac{\partial}{\partial y} I_0 \left(2\sqrt{ABxy}\right). \tag{64}$$

For sufficiently low flow rate, we may use only the first term of the asymptotic expansion of $I_0$ and find

$$\frac{\partial\,(c/c_0)}{\partial y} = \frac{A}{2\sqrt{\pi}} \frac{e^{-(\sqrt{Bx} - \sqrt{Ay})^2}}{(ABxy)^{\frac{1}{4}}} \left\{\sqrt{\frac{Bx}{Ay}} - \frac{1}{4Ay}\right\} \tag{65}$$

which for $Bx = Ay$ reduces to

$$\textit{Slope at Mid-Point} \simeq \frac{1}{2\sqrt{\pi}} \cdot \frac{1}{y} \left\{\sqrt{Ay} - \frac{1}{4\sqrt{Ay}}\right\} \tag{65a}$$

Thus, we have a means of finding $A$ and, hence, $k_2$.

In the case of Langmuir kinetics, it is apparent, from EQUATION 62, that the center of the saturation curve will give a measure of $K$. In this case, since an experimental equation for the isotherm enables one to determine both $K$ and $a$, one can find values for both velocity constants. Only in the limiting case in which EQUATION 62 is applicable can a very simple expression for the slope of the saturation curve be obtained. One finds easily

$$\text{Slope at Mid-Point} \simeq \frac{1}{4}\frac{k_1 c_0}{\dot{V}} \tag{66}$$

The applicability of EQUATION 66 will probably prove to be limited. It can be valid only for very slow flow; in these cases, the curves will be steep and the slopes difficult to determine with any precision.

The methods of this paper have thus far been applied only in a limited number of cases. With more experience and more experiments, some more generally satisfactory means of evaluating the velocity constants can, no doubt, be developed.

[**Note.** During the preparation of this paper, the contribution on the same subject of L. G. Sillen[16] has come to the author's attention. Sillen has treated the problem in an essentially identical manner. He has found solutions of the differential equations for several special cases. It is of interest to notice that his solutions turn up as particular cases of the general solutions of this paper. Thus, Sillen's Equations 13 are identical with our EQUATIONS 52 and 53 if, in our equations, we put $k_2 = 0$. Similarly, the solution of Sillen by his "method of final functions" for the general case of Langmuir kinetics, his Equation 35, is identical with our limiting EQUATION 62.

The complete solution of the ion-exchange problem considered by Sillen and Ekedahl[17] has been given.[10]]

## EXPERIMENTAL PART

To gain information as to the applicability of the kinetics at the basis of the formulae established under the heading of "Theory", experiments have been carried out on the adsorption of anthracene from cyclohexane solution flowing over activated alumina. Provision was made for varying the rate of flow. Analysis of effluent solutions was carried out spectrophotometrically. It should be emphasized at once that these experiments are of a preliminary nature. Although great care was taken to obtain results of as good accuracy as was possible to the experiment, the design of this experiment was deliberately simplified. Quantitatively, the results cannot be said to be of fundamental significance and are not intended to be so taken. The chief simplification of the experiment was the manner in which the adsorbent was handled. The material was merely taken from a large bottle of commercial activated alumina and introduced directly into the column. In a careful experiment, all precautions should be taken to secure reproducible activity of the adsorbent. Furthermore, in the present experiments, from the nature of the apparatus used, there may be some uncertainty in the total volume of solution passed, due to uncertainty in the volume of dead spaces at both the input and exit ends of the tube. Thus, the absolute positions of the mid-points of the various curves are subject to some error. As has been shown, the positions of these points are of importance in the discussion of the results.

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[16] **Sillen, L. G.** Arkiv. Kem. Min. och Geol. **22A** (15): 1. 1946.
[17] **Sillen, L. G., & E. Ekedahl.** Arkiv. Kem. Min. och Geol. **22A** (16): 1. 1946.

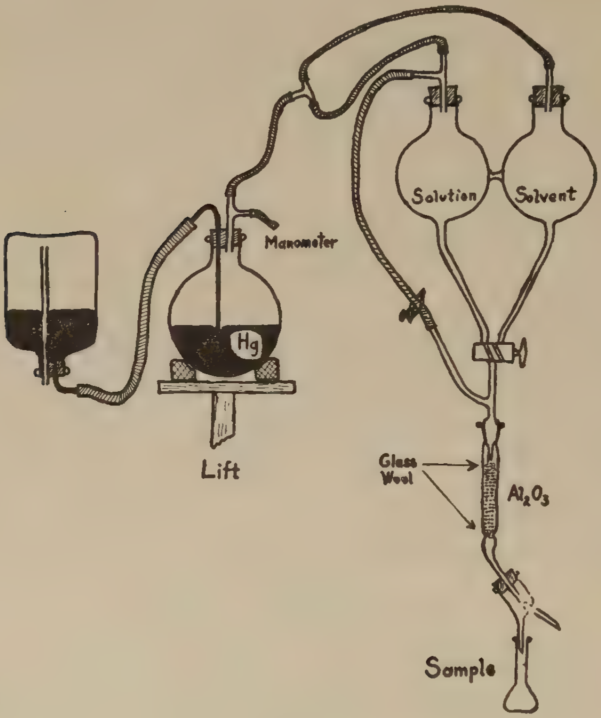

FIGURE 1.

**Method and Results.** The sketch of FIGURE 1 shows the apparatus used. The pressure was maintained by manual adjustment of the mercury levels in the siphon. The selector stopcock was very carefully ground; no grease was used. The column was filled by sprinkling the alumina into the tube filled with cyclohexane. The alumina was supported on a light wad of pyrex glass wool which had been carefully shaped to give the column as flat a base as possible. The columns were conditioned by forcing a hundred or more milliliters of solvent through them before the experiment was started. Only after such treatment was the rate of flow determined by the applied pressure. During an experiment, the flow rate was maintained constant to better than five per cent.

The entire effluent of the column during an experiment was taken as successive 5, 10, or 25 ml. samples. All solution preparation and sampling were done by weight. Volumes and concentrations were calculated using 0.778 for the density of the solutions.

The experiments were carried out in a temperature-regulated room, no further attempt at temperature control being made. The room temperature varied no more than a degree from 22°C.

A very pure sample of anthracene was used. The material had been codistilled two or three times with ethylene glycol and washed thoroughly with water. It showed the blue fluorescence characteristic of the pure compound. Commercial cyclohexane was treated with fuming sulfuric acid, washed, dried, distilled, and stored over sodium. For the preparation of these materials I am much indebted to Professor Harold G. Cassidy of this Laboratory. The alumina, as mentioned already, was taken directly from a bottle of 80-200 mesh material.

A Beckman spectrophotometer set at a wavelength of 367 m$\mu$ with a slit width of 0.079 mm. was used in the analysis of the cyclohexane solution of anthracene. The method is very reliable in the range of 0-0.01 weight percent. As is shown in FIGURE 2, Beer's law is strictly followed in this region. Twenty solutions, prepared from several different stock solutions, varying from 0.000907% to 0.00999%, were used in the calibration. In terms of concentrations (mmol./ml.) the results are well represented by the straight line

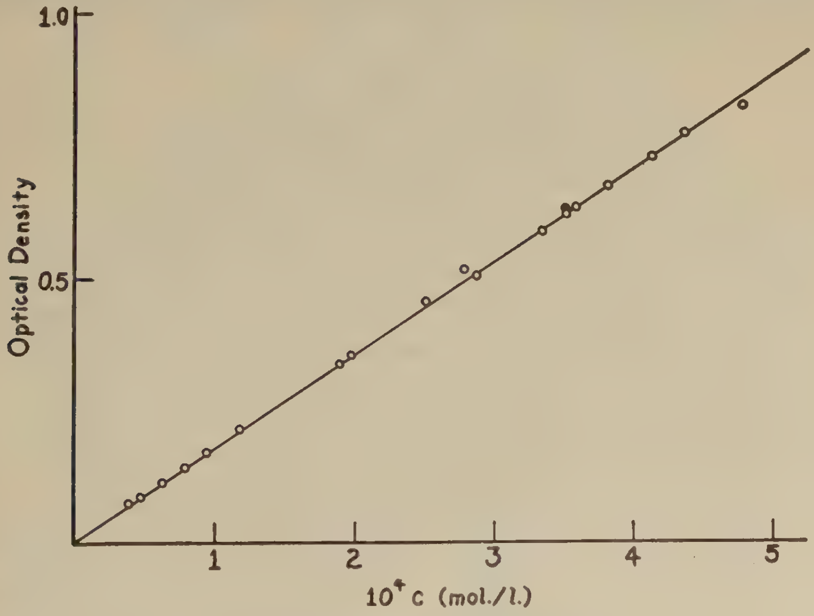

FIGURE 2. Spectrophotometer calibration.

$$c = 5.63 \times 10^{-4}\, d,$$

in which $d$ is the "optical density" as read directly from the instrument. Except for the most dilute solutions, it is considered that the individual analyses are accurate to 2 per cent or better. Since the concentrations of the input solutions were determined by weight, the ratios $c/c_0$ are subject to analytical errors no greater than this value.

A determination of the free space of the alumina packed in cyclohexane gave $m = 1.37$ ml./g. Since volumes *through* the column were measured, $m$ does not enter into any of the theoretical computations of the results. The diameter and lengths of the columns are in this same category of unnecessary information. The diameter of the column was 0.82 cm. In the two experiments here reported, the average lengths were 4.79 ± 0.09 cm. and 4.86 ± 0.04 cm., respectively. The weight of alumina in the column was determined by weighing the entire column after the experiment, the solvent having been removed by a current of air.

The results obtained for two complete liquid chromatograms are given in TABLES 1 and 2. These data were obtained using solvent from a single bottle. Unfortunate experience showed that anomalous results might be obtained by using material from slightly different sources. In one elution experiment, the column was saturated using a solution prepared with

TABLE 1
LIQUID CHROMATOGRAM
Anthracene-cyclohexane-alumina at 22° C
Weight of $Al_2O_3$, $x = 2.316$ g.
Average rate of flow: Saturation = 5.0 ml./min.
Elution = 5.4 ml./min.
Initial concentration, $c_0$ = 0.000811 mmol./ml.

| Saturation | | Elution | |
|---|---|---|---|
| Vol. ($\Delta y$) ml. | $c/c_0$ | Vol. ($\Delta y$) ml. | $c/c_0$ |
| 9.96 | 0.002 | 10.06 | 1.009 |
| 9.99 | .003 | 5.04 | .990 |
| 10.16 | .006 | 4.94 | .924 |
| 5.06 | .038 | 4.95 | .839 |
| 5.09 | .121 | 5.12 | .734 |
| 5.12 | .245 | 4.56 | .643 |
| 4.91 | .403 | 5.12 | .558 |
| 9.99 | .625 | 10.05 | .431 |
| 10.14 | .826 | 10.04 | .319 |
| 9.97 | .925 | 10.04 | .237 |
| 10.00 | .971 | 10.07 | .183 |
| 10.01 | .987 | 9.96 | .141 |
| 10.07 | 1.000 | 10.01 | .112 |
| 10.01 | .998 | 10.04 | .087 |
| 10.07 | 1.006 | 25.01 | .062 |
| 9.88 | 1.003 | 25.08 | .038 |
| | | 25.01 | .022 |
| | | 25.03 | .017 |
| | | 25.05 | .012 |
| | | 25.12 | .007 |

cyclohexane from one container and eluted with supposedly identical material from another bottle. The initial effluent came through at a much higher concentration than that of the saturating solution, indicating a large change in the character of the adsorption. As a matter of fact, this effect made it necessary to discard the results of several weeks' work. It was impossible to show that the solvent was essentially identical for all the experiments. This sensitivity to very slight impurities in the solvent is another reason why the present results must not be taken as absolute determinations.

**The Isotherm.** FIGURE 3 contains the results of experiments designed to determine the adsorption isotherm of the system. To weighed flasks equipped with carefully ground stoppers were added, in succession, alumina and appropriate amounts of cyclohexane and stock solution of anthracene in cyclohexane. (All solvent was from the same sample used in determining the chromatograms reported.) After fifteen minutes, during which time the flasks were vigorously shaken at frequent intervals, samples were withdrawn through plugs of cotton wool. Experiments showed that fifteen minutes was ample time for complete reaction. The

TABLE 2

LIQUID CHROMATOGRAM

Anthracene-cyclohexane-alumina at 22° C

Weight of $Al_2O_3$, $x = 2.316$ g.

Average rate of flow: Saturation = 9.8 ml./min.

Elution = 9.9 ml./min.

Initial concentration, $c_0 = 0.000811$ mmol./ml.

| Saturation | | Elution | |
|---|---|---|---|
| Vol. ($\Delta y$) ml. | $c/c_0$ | Vol. ($\Delta y$) ml. | $c/c_0$ |
| 10.09 | 0.000 | 4.97 | 1.003 |
| 5.10 | .003 | 5.04 | .995 |
| 5.04 | .004 | 5.02 | .941 |
| 5.06 | .025 | 5.05 | .861 |
| 5.07 | .071 | 5.04 | .771 |
| 10.04 | .184 | 4.93 | .678 |
| 10.02 | .383 | 10.04 | .561 |
| 10.04 | .582 | 10.05 | .432 |
| 10.11 | .737 | 10.04 | .332 |
| 10.02 | .839 | 10.02 | .264 |
| 10.07 | .905 | 10.00 | .209 |
| 9.98 | .948 | 10.02 | .167 |
| 10.04 | .977 | 9.94 | .138 |
| 9.97 | .983 | 10.05 | .112 |
| 10.06 | .994 | 10.03 | .093 |
| 23.31 | 1.002 | 25.11 | .070 |
| | | 24.95 | .047 |
| | | 25.03 | .032 |
| | | 25.08 | .023 |
| | | 25.06 | .017 |
| | | 25.00 | .012 |
| | | 25.00 | .010 |

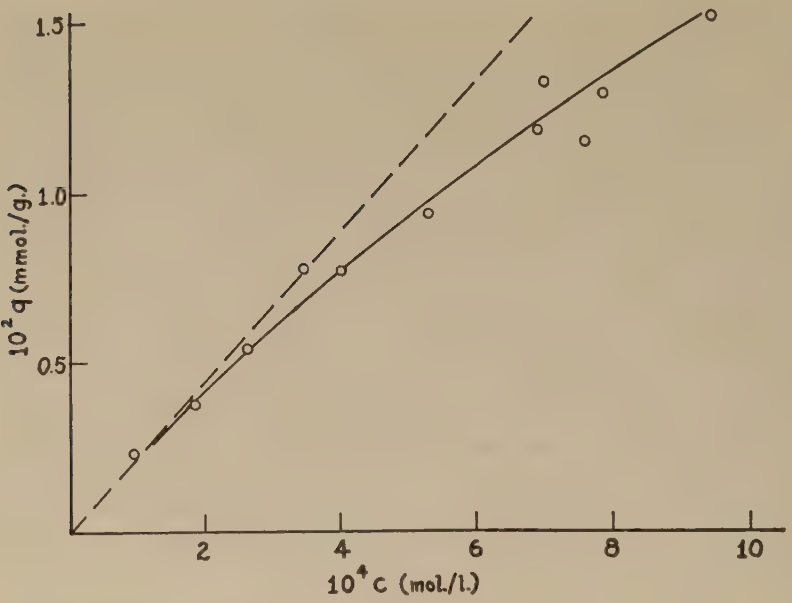

FIGURE 3. Adsorption isotherm $C_{14}H_{10}$ in cyclohexane on $Al_2O_3$.

cotton plugs in the pipettes were shown to be without effect on the concentration of the samples. Analysis was made in the spectrophotometer. As is apparent, the accumulation of errors involved in determinations of this kind produces large effects on the reproducibility of the results. The solid line of FIGURE 3 is the plot of the Langmuir isotherm,

$$q = \frac{22\,c}{1 + 375\,c}$$

while the dashed line is the plot of the linear isotherm,

$$q = 22\,c.$$

**Discussion.** It has not been thought worth while to attempt the application of the complete theory for Langmuir kinetics to the experimental results here given. We will content ourselves, at present, with a tentative discussion using the linear kinetics, largely because of the greater simplicity of the computations in this case. After considerable preliminary computation, it has been found that on this basis the results of TABLES 1 and 2 can, perhaps, best be represented if we choose for the constants of the theory,

$$k_1 a = 59.5,$$

$$k_2 = 2.71.$$

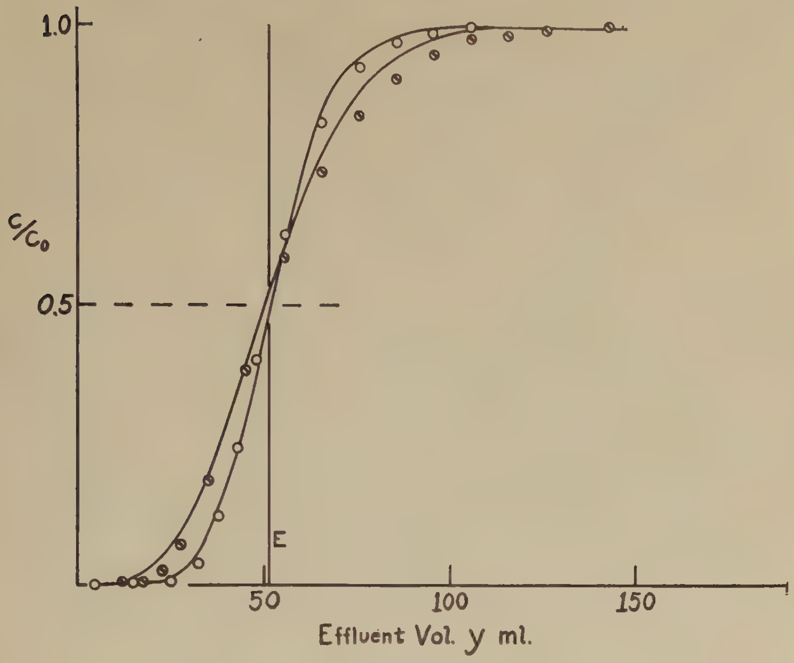

FIGURE 4. Saturation curves $C_{14}H_{10}$ in cyclohexane on $Al_2O_3$. O=5.0 ml./min., Θ=9.8 ml./min. Lines from linear theory, $k_1a=59.5$, $k_2=2.71$; E=equil. theory; $q=22c$.

This choice is consistent with the linear isotherm, $q = 22\ c$. In FIGURES 4 and 5, the computed results are given in the form of curves. The experimental points here are plotted against the average $y$ for the particular sample. In the case of the saturation curves, the agreement with the data is better than qualitative. The reproduction of the general character of the results is striking. The quantitative disagreement is, however, in most cases (particularly in the more concentrated solutions toward the upper portions of the curves) certainly outside the limits of a reasonable estimate of the experimental error. In the case of the elution curves of FIGURE 5, we have only qualitative agreement. The theoretical curves lie in the correct order, have the proper general shape, but in no way quantitatively reproduce the data. (They do, however, represent an improvement over the curve given by the equilibrium theory, which is also depicted in FIGURE 5.) In what part these discrepancies are due to fundamental inadequacies of the theory and in what part to overly simplified computation procedure, cannot at present be decided. The first requirement toward making this decision will be more and better experimentation. More experiments are planned for the near future. It is hoped that the theory will also receive testing at the hands of others.

**Note on the Theory for Multiple Solutes.** The simplest way in

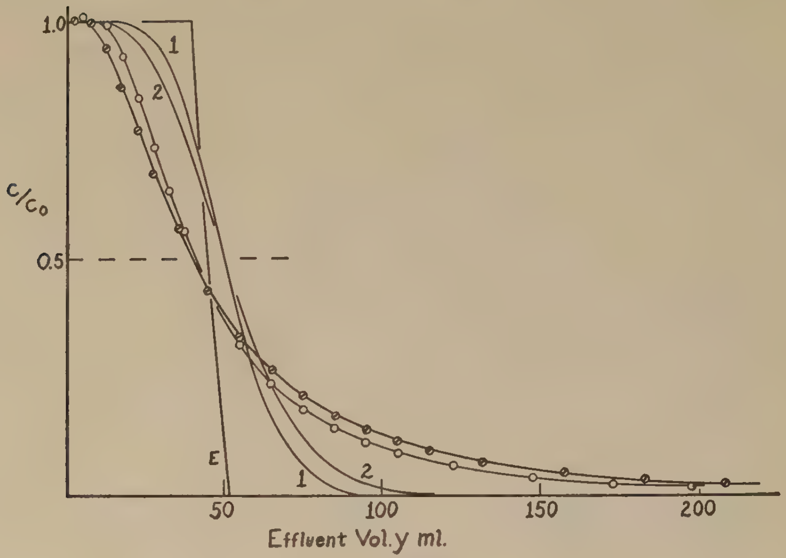

FIGURE 5. Elution curves, $C_{14}H_{10}$ in cyclohexane on $Al_2O_3$. ○=5.4 ml./min., ⊖=9.9 ml./min. 1 and 2 (same rates) by linear theory, $k_1a=59.5$, $k_2=2.71$; E equil. theory, $q=22c/(1+375c)$.

which interaction during adsorption of two or more solutes can be represented is by neglecting all mutual chemical effects and supposing that the availability of sites on the adsorbent is the determining factor. Again, we predicate the simplest kinetics leading to a Langmuir adsorption isotherm. The differential equations for the corresponding rate processes in the two solute case are,

$$\frac{\partial^2 F_1}{\partial x\,\partial y} + A_1\frac{\partial F_1}{\partial x} + B_1\frac{\partial F_1}{\partial y} + C_1\left\{\frac{\partial F_1}{\partial x}\frac{\partial F_1}{\partial y} + \frac{\partial F_2}{\partial x}\frac{\partial F_1}{\partial y}\right\} = 0,$$

$$\frac{\partial^2 F_2}{\partial x\,\partial y} + A_2\frac{\partial F_2}{\partial x} + B_2\frac{\partial F_2}{\partial y} + C_2\left\{\frac{\partial F_2}{\partial x}\frac{\partial F_2}{\partial y} + \frac{\partial F_1}{\partial x}\frac{\partial F_2}{\partial y}\right\} = 0.$$

The mathematical problem thus posed is most formidable. There seems to be reasonable doubt that the complete solution of such a non-linear system can be obtained by the methods of contemporary mathematics. Possibly, some trick might be found which would reduce the equations to tractable form. After more knowledge of single solute cases is gathered, something might be done with the two-solute case by numerical methods. Presumably, the equilibrium theory of Glückauf[9] forms the limiting cases for slow flow. It is to this theory and to experiment that one must turn for information on what is, after all, the most important case of chromatography, the separation of two substances.

# FRONTAL ANALYSIS AND DISPLACEMENT DEVELOPMENT IN CHROMATOGRAPHY

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## INTRODUCTION

In this paper, a description will be given of the arrangements for chromatographic adsorption analysis which have been worked out in Uppsala by Tiselius and his co-workers[1-20].* The basic principle behind the method is liquid chromatography, which means that the whole process of separation is followed by measuring the concentration of the solution leaving the column and not, as in ordinary chromatography, by looking at the zones on the column. The experimental arrangement is given schematically in FIGURE 1. The solution is poured into the vessel *A*, forced through the filter with adsorbent *B*, and the concentration of the solution leaving the filter is observed in the small cell *C*. It is essential that the cuvette *C* should have a very small volume, so as to avoid lag in the readings and to provide for the swift replacement of solution in the cuvette, thus eliminating possible convections. The concentration in the

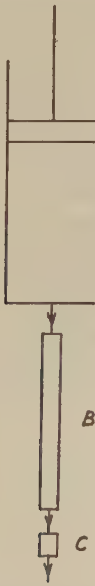

FIGURE 1. Diagram of apparatus for adsorption analysis.

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* A detailed description of the whole subject can be found in Claesson, S. Studies on adsorption and adsorption analysis with special reference to homologous series. Arkiv Kemi, Mineral., Geol. 23A, 1: 1–133. 1946.

cell *C* is then plotted against the volume of solution that has passed through the filter *B*. In this way, characteristic curves are obtained from which the qualitative and quantitative composition of a mixture can be calculated.

This procedure has several advantages over the older chromatographic method:

(1) It is especially suited for analysis of colorless substances, and is independent of the color of the adsorbent.

(2) The separation of the different components is much greater in the solution leaving the filter than in the column, which means that the selectivity increases considerably.

(3) The procedure is especially suited for quantitative evaluations of the adsorption phenomenon in the column. It is also clear that, by a proper choice of the method for the determination of the concentration in the cell *C*, it is possible to adapt this method of adsorption analysis for almost all systems met with in practice. In all experiments reported in this paper, the concentration is followed by measurement of the change in the refractive index ($\Delta\mu$) for solutions and the change in the thermal conductivity ($\Delta\lambda$) for gases.

## THE INTERPRETATION OF THE DIAGRAMS

When the method for chromatographic adsorption analysis schematically given in FIGURE 1 is used, the experiments can be arranged in three different ways, namely, frontal analysis, elution analysis, and displacement development. A short review of these three adaptations will be given here. For a more detailed description, the reader is referred to Claesson.[18]

**Frontal Analysis.** Frontal analysis is the simplest method of adsorption analysis and is carried out as follows. The filter with adsorbent is at first washed with pure solvent, after which the solution to be analyzed is poured into the vessel *A* (FIGURE 1) and is allowed to pass the filter *B*. The solute is adsorbed and moves forward with a sharp front as the filter becomes saturated. At the beginning, pure solvent comes through the cell *C*, and the concentration of the solute is zero. When the front has passed the whole filter (that is, when the adsorbent is completely saturated), the concentration in the cell *C* suddenly increases. Diagrams with one step (FIGURE 2) are thus obtained when there is only one solute, with

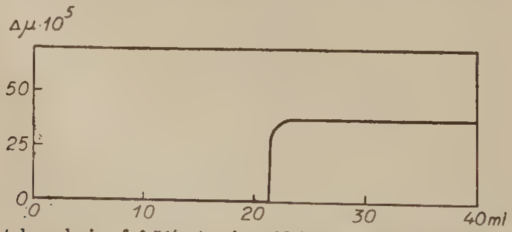

FIGURE 2. Frontal analysis of 0.5% stearic acid in ethanol. Filter: 1250π carboraffin C.

two steps when there are two solutes (FIGURE 3) and so on. The first step contains component 1, the second step component 1 *and* component 2, and similarly the *i*th step components 1, 2, 3, . . . *i*-1 and *i*. The volume that has passed the filter before the solute *i* breaks through was called the *retention volume* for the component *i*, by Tiselius. This volume includes, of course, the small volume of solution necessary to replace the solvent between the adsorbent particles in the filter at the beginning of the experiment. After subtracting this small volume from the retention volume, the *corrected retention volume* $v_i$ is obtained. If the corrected retention volume is divided by the weight of the adsorbent in the filter, the *specific retention volume* $v_i^0$ (corrected retention volume per gram adsorbent) is obtained.

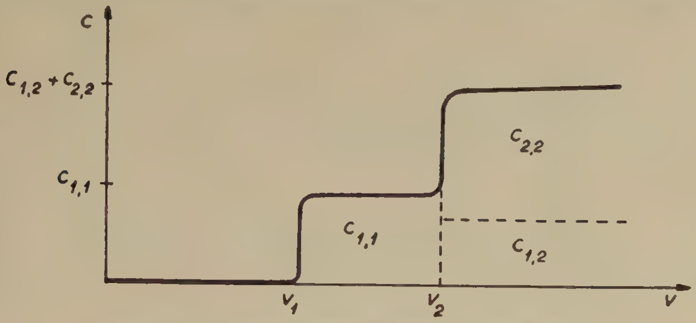

FIGURE 3. Frontal analysis diagram for two solutes.

For a system of one solute, the amount of substance $a$ adsorbed in the filter has previously been dissolved in the corrected retention volume $v$, since, after the breaking through of the front, the solution passes unchanged through the filter. If $c$ is the concentration of the solution, we get

$$a = v.c \tag{1}$$

or, per gram adsorbent,

$$a^0 = v^0.c \tag{1a}$$

When the adsorbed amount $a^0$ is plotted against the concentration $c$, the adsorption isotherm $f(c)$ for the substance in question is obtained, as the amount adsorbed $a^0$ is in equilibrium with the solution of concentration $c$. Thus, we have

$$a^0 = f(c) = v^0.c \tag{2a}$$

As the adsorption isotherm normally has the shape given in FIGURE 5, the specific retention volume

$$v^0 = \frac{f(c)}{c} \tag{2a}$$

will decrease as the concentration $c$ increases. When the concentration tends to zero, the specific retention volume will assume a constant value equal to the slope of the first linear part of the isotherm.

When frontal analysis is carried out on a solution containing two solutes, two steps will appear in the diagram (FIGURE 3). The solution between the two steps contains only solute 1, but its concentration $c_{1,1}$ is higher than in the original solution $c_{1,2}$. This is due to the fact that, as the second front (component 2) moves through the filter, a certain amount of component 1 is displaced from the adsorbent, and appears in the first step.

It is easily seen that, if an experiment is carried out with a solution of known concentrations $c_{1,2}$ and $c_{2,2}$, we have the following expressions for the amounts adsorbed.

$$a_1 = v_2 \cdot c_{1,2} - (v_2 - v_1) \cdot c_{1,1}$$
$$a_2 = v_2 \cdot c_{2,2}$$

or, per gram adsorbent,

$$a_1^o = v_2^o \cdot c_{1,2} - (v_2^o - v_1^o) \cdot c_{1,1} \qquad (3)$$
$$a_2^o = v_2^o \cdot c_{2,2}$$

In this way, it is very easy to study mixed adsorption of systems containing two solutes. In the case of three or more solutes, it is not possible to calculate the amounts adsorbed from the frontal analysis diagram, since there are more unknowns than equations.

However, the important thing, from the analytical point of view, is to be able to calculate the concentrations of an unknown solution from the diagram, and that can only be done if the equations of the adsorption isotherms are known. As it is essential to avoid too complicated calculations, a simple approximate formula for the isotherm must be used. It is always possible to carry out all the experiments at the same total concentration, and so even a rough approximation of the isotherm will give rather good accuracy. By using Langmuir's equation for the isotherm,

$$a_i^o = \frac{k_i c_i}{1 + l_i c_i} \qquad (4)$$

especially simple formulae can be derived. (Here, $a_i^o$ is the amount adsorbed per gram adsorbent of component $i$ with the concentration $c_i$; $k_i$ and $l_i$ are constants).

In case of two solutes, the formula will be (FIGURE 2):

$$c_{1,2} = c_{1,1} \cdot \frac{1 - \dfrac{v_1}{v_2}}{1 - \dfrac{k_1}{k_2}} \qquad (5)$$

The correct value for the concentration of component 1 ($c_{1,2}$) is thus obtained directly, as $c_{1,1}$, $v_1$, and $v_2$ are easily measured. The concentration, $c_{2,2}$ of component 2 is then obtained by subtraction from the total concentration $c_{1,2} + c_{2,2}$.

In case of $n$ solutes, EQUATION 5 will take the more general form

$$c_{i,m+1} = c_{i,m} \frac{1 - \frac{k_i}{k_m} \cdot \frac{v_m}{v_{m+1}}}{1 - \frac{k_i}{k_{m+1}}} \tag{6}$$

where $c_{i,m}$ is the concentration of component $i$ in the $m$th step. Consequently, all the concentrations in the diagram can be calculated successively. For the derivation of these formulae, the reader is referred to Claesson.[18]

The expressions obtained are strikingly simple,* and it is remarkable that the constants $l_i$ do not appear, but only $k_i$, which in homologous series, for example, can be determined by Traube's rule. There, we can put

$$k_n = p . q^n, \tag{7}$$

where $p$ and $q$ are constants, and $n$ represents the number of carbon atoms in the compound. In this way, the calculations are very much simplified, and only two constants $p$ and $q$ need be known for a whole homologous series. Furthermore, a rough estimation of the value $k_n$ can be obtained from the diagram by means of a formula (Claesson[18])

$$k_n = v_n^0 \, (1 + l_1 c_{1,n} + l_2 c_{2,n} + \ldots .) \tag{8}$$

Then it is possible to calculate $n$, the number of carbon atoms in the component, by EQUATION 7 and, thus, a qualitative analysis is achieved.

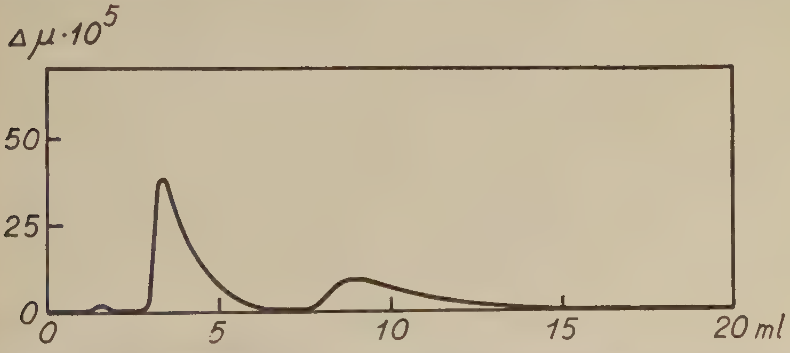

FIGURE 4. Elution analysis of 0.5 ml. of a solution containing 5 mg. each of lauric and palmitic acids. Filter: 500π carboraffin CI. Solvent (also used for elution) absolute ethanol. The small peak to the left is due to water in the ethanol.

**Elution analysis.** This procedure is identical with that of ordinary chromatographic analysis. After having washed the filter with pure solvent, a small volume of the solution to be analyzed is forced into the top of the column. The vessel $A$ is then filled with pure solvent which is allowed to pass through the filter. Thus, the concentration of the solute in the cuvette $C$ is zero during the course of the experiment, except when the different zones are moving through it. The resulting diagrams are shaped as in FIGURE 4, where each component is represented by a peak. The area $A$ under a peak gives the amount of substance according to the equation

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* It can, in fact, be shown that the simple EQUATION 6 is obtained if the equation of the adsorption isotherm can be put in the form $a_i^0 = k_i c_i . r(c_1, \ldots c_n)$, where $r$ is an arbitrary function but, of course, the same for all the components. This condition is necessary and sufficient (**Claesson**[20]).

$$A = \int_{v_1}^{v_2} c\,dv. \tag{9}$$

A quantitative analysis of the mixture is thus obtained by the simple measurements of areas. As every component comes out in a pure state, it can be identified by ordinary analytical methods. This means that the elution process is well adapted for preparative purposes, an advantage over the frontal analysis where only the weakest adsorbable component can be collected in a pure state. However, we have the disadvantage of the long "tails" of the peaks representing strongly adsorbed substances, which renders their separation difficult and makes the determination of the area $A$ uncertain.

**Displacement Development.** This very ingenious method for adsorption analysis was introduced by Tiselius.[8] It eliminates all the disadvantages of elution analysis and is performed as follows. In the same way as in elution analysis, a small volume of the solution to be

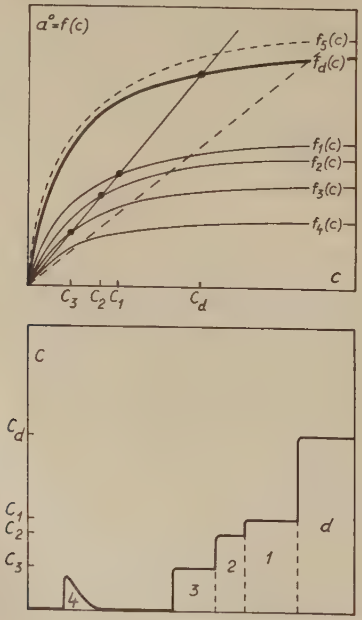

FIGURE 5. The construction of the displacement development diagram from the adsorption isotherms.

analyzed is forced into the top of the column, but instead of using pure solvent the column is afterwards washed with a solution of a substance, "developer", which is more strongly adsorbed than any of the components in the mixture. In fact, a frontal analysis of the developer is performed behind the mixture to be analyzed. By displacement, the components of the mixture are forced to move in front of the front of the developer. The components will also displace one another, so that they are arranged in order of increasing adsorption affinity, the one with the strongest adsorption affinity moving as a band immediately in front of the front of the developer (FIGURE 5). All the different components are thus obtained in pure state and the method is, therefore, also well suited for preparative purposes.

It is easily seen that, as soon as a stationary state has been established, all the zones in the column move forward with the same speed as the front of the developer. The latter has a speed which depends on its specific retention volume $v_d^o$. As each of the zones contains only a single component, we can apply EQUATION 2a and get

$$\frac{f_d\,(c_d)}{c_d} = \frac{f_1\,(c_1)}{c_1} = \frac{f_2\,(c_2)}{c_2} = \ldots . = v_d^o \qquad (11)$$

If the concentration of the developer $c_d$ is kept constant in all experiments, then EQUATION 10 will give the values of the concentrations of all the components 1, 2, 3, . . . in the diagram. EQUATION 10 can easily be solved graphically. In the diagram showing the isotherms (FIGURE 5), a straight line is drawn from the origin to the point on the isotherm of the developer corresponding to the concentration $c_d$. The equation of the line will be

$$a^o = f(c) = v_d^o.c, \qquad (11)$$

and the intersections of that line and the corresponding isotherms will define the equilibrium concentrations $c_1$, $c_2$, $c_3$, . . . From the figure, it is also evident that, if one component, 4, is adsorbed so weakly that the isotherm does not intersect the straight line, no equilibrium concentration is obtained. The component moves so rapidly through the filter that it cannot be overtaken by the developer, and it appears in the diagram as a free peak. By increasing the concentration $c_d$ sufficiently, component 4 will also be displaced (dashed line in FIGURE 5).

From the foregoing, it is evident that, if the concentration $c_d$ of the developer is kept constant in all experiments, the height of a step is independent of the amount of substance present in the step and only dependent of the nature of the substance. This *specific height, h,* is determined once and for all and is then used for the qualitative identification of the substance. As the area under a step is proportional to the amount of substance present and the height at the step is constant, the length of the step must be proportional to the amount of substance. When the proportionality factor $b$ (length of step per gram of substance, *specific length*) is determined once and for all, the quantitative composition will be obtained by dividing the length of the steps by $b$.

For displacement development, we have thus obtained the important result that (1) the qualitative analysis is obtained by measurements of the heights of the steps, and (2) the quantitative analysis is obtained by measurements of the lengths of the steps. Displacement development is, therefore, the best method for adsorption analysis, but, unfortunately, it is not always applicable. Certain substances are adsorbed irreversibly and can neither be displaced nor eliminated, which leaves frontal analysis as the only possible way.

## THE APPARATUS AND EXPERIMENTAL ARRANGEMENTS

The most important problem in the construction of an apparatus for adsorption analysis is the choice of a suitable method for the continuous determination of the concentration. In order that the method may apply as generally as possible, it is necessary to measure a property of the solution independent of the specific properties of the solvent and the solute. For that reason, the determination of refractive index was selected as most suitable. It is quite clear, however, that, in many special cases, a more specific and sensitive method may be used with greater advantage, as it is hardly possible to use refractive index determinations for solutions with a concentration smaller than 0.01%.

It is quite evident that the method for adsorption analysis discussed in the preceding section is also applicable in the case of gaseous systems, provided that the gaseous mixture to be analyzed is mixed with an inert gas, *e.g.*, nitrogen, which acts as a solvent. Therefore, an apparatus for adsorption analysis of gases and vapors will also be described.

**Apparatus for Liquids.** Two pieces of apparatus will be described; one, an interferometer which gives very high accuracy and is used for analytical purposes; the other, a self-recording apparatus with slightly lower sensitivity, which is used for preparative experiments and for analytical work on moderately dilute solutions.

The filter for the adsorbent and the container for the solution are of the same type for both pieces of apparatus. The adsorbent is packed in cylindrical tubes of brass, gold-plated inside and nickel-plated outside. The adsorbent rests on filter paper supported by perforated disks of metal. Filters of many different sizes have been used, the smallest having an inside diameter of 4 mm. and a height of 12.5 mm., the largest a diameter of 40 mm. and a height of 100 mm. For classifying the filters, their volume in $mm.^3$ has been used. In order to obtain simple numbers, the factor $\pi$ has not been taken into account. The two filters mentioned above are, consequently, called $50\pi$ and $40{,}000\pi$, respectively. Four filters ($50\pi$, $250\pi$, $500\pi$, and $1250\pi$) are shown in FIGURE 6. The filters are attached to the container for the solution by means of a heavy nut.

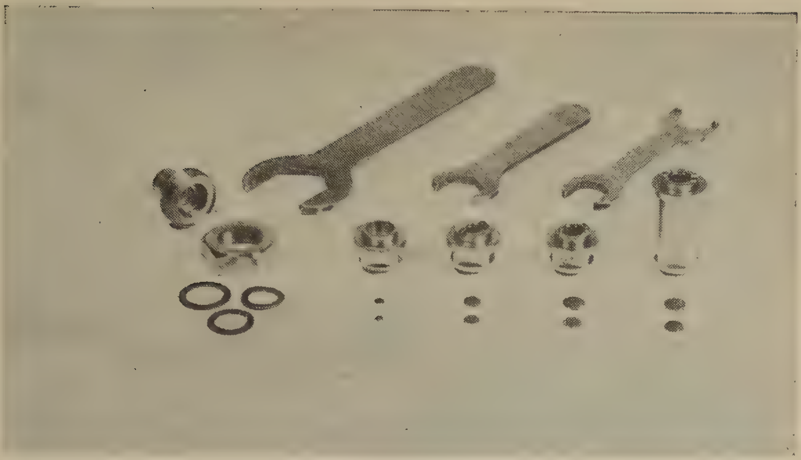

FIGURE 6. Filters for adsorbent.

When water is used as a solvent, the container for the solution is an ordinary tube of glass (FIGURE 7a) with fittings for the filter and for the rubber tubing from a pressure tank (ca. 3 kg./cm.$^2$). This pressure gives the solution a suitable speed through the filter (ca. 0.5 ml./min.). When

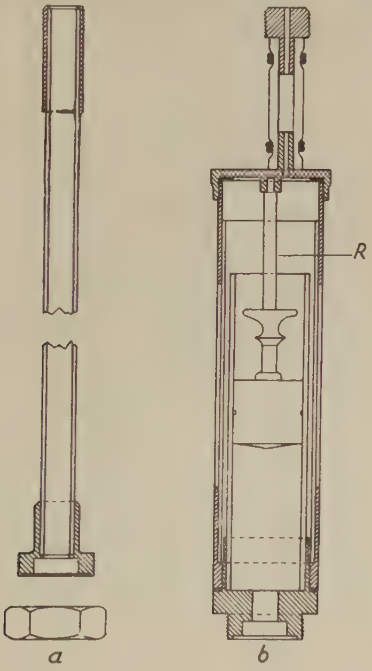

FIGURE 7. Containers for the solution.

organic solvents are used, it is necessary to prevent the air from the pressure tank being dissolved in the solution, as this would affect the refractive index. The air-free solution is therefore filled in a syringe (FIGURE 7b) enclosed in a metal tube. Then the air pressure acts on the piston and no air is dissolved in the solution.

**The Interferometer.** The interferometer is of the Rayleigh-Haber-Löwe type[21] and has been so designed that it is possible to use a very small volume of solution for the measurement. The cell consists of a cylindrical hole in a block of brass. The length of the hole is 80 mm. and its diameter 1.4 mm. The filter with adsorbent is screwed into the cuvette and the solution flows through the cuvette and is then collected in a rack of graduated tubes where the volume of solution is measured. FIGURE 8 shows the cuvette mounted together with the syringe for the solution and a 1250$\pi$ filter. There are four holes in the block: one for the solution, and the other three, reference cells filled with pure solvent. The cuvette is placed in a double thermostat. The light source is a tungsten ribbon lamp giving white light. The compensator is of the usual design, a plane plate of glass being turned by a micrometer

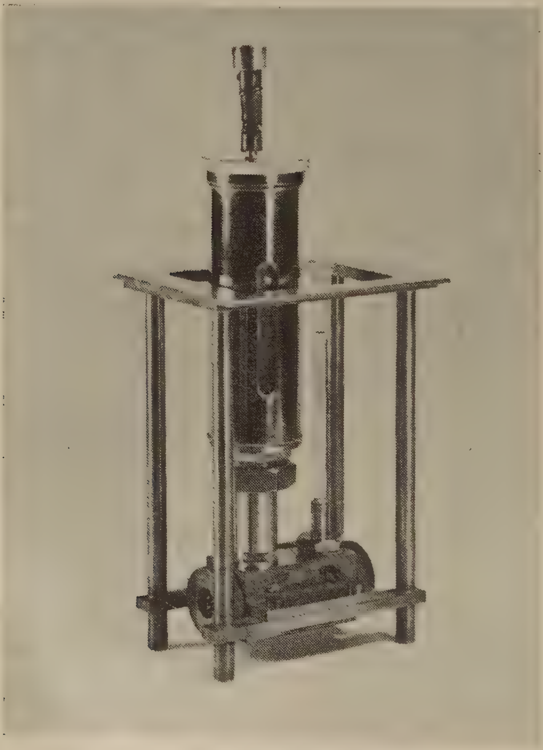

FIGURE 8. Interferometer cuvette, filter, syringe, and outlet tube mounted together.

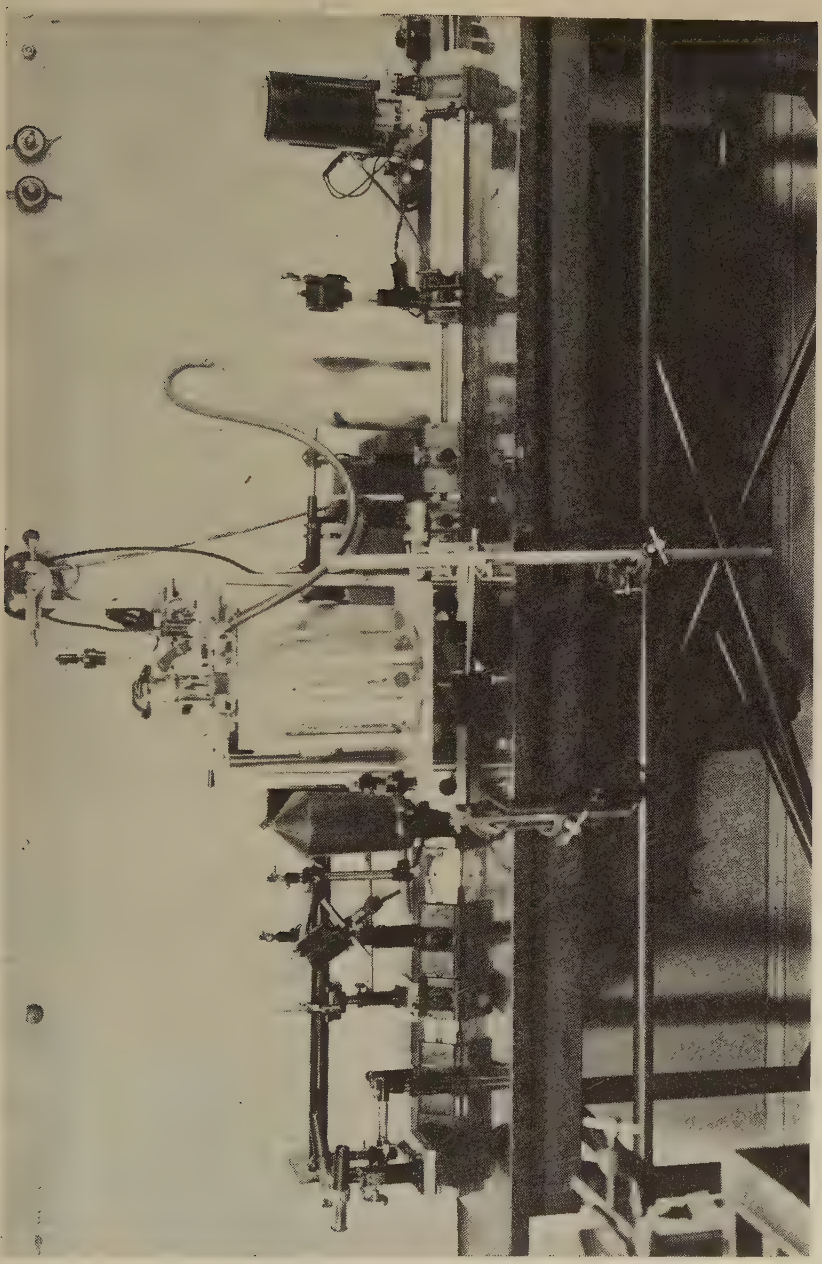

FIGURE 9. Photograph of the interferometric arrangement for adsorption analysis.

screw acting on a long arm. When measurements are performed on flowing solutions, the accuracy of reading amounts to $5.10^{-6}$ in refractive index and the total region of measurement is $6.10^{-3}$. The values are obtained from the position of the micrometer screw, which has been calibrated once and for all by counting interference fringes in monochromatic light.

FIGURE 9 is a photograph of a complete apparatus. The rack with the test tubes is seen under a bell jar in front of the thermostat in the middle of the figure. To the left are the ocular and two small telescopes, one being used for the reading of the micrometer screw, the other for the reading of the volume in the graduated test tubes.

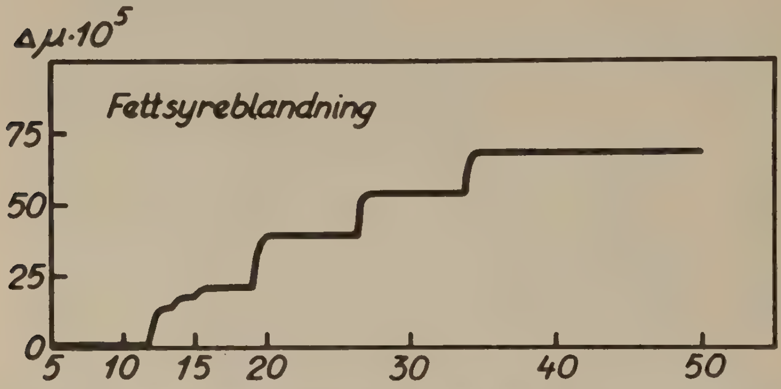

FIGURE 10. Frontal analysis of a mixture of six fatty acids (caprylic, pelargonic, capric, lauric, myristic, and palmitic acids). See TABLE 1.

FIGURE 10 shows a typical diagram obtained with this apparatus.

**The Self-Recording Apparatus.** In this apparatus, the solution leaving the filter with adsorbent is passing one-half of a double hollow prism (*D*, FIGURE 11). The other half of the prism is filled with pure solvent. A beam of light passes the prism and makes a deflection which is proportional to the change in refractive index. This deflection is very small (about 0.01 mm. if $l = 1000$ mm.) and is magnified in the following way. The beam of light is divided in two parts by a hexagonal prism of glass *P*, which fall on photoelements ($C_1$ and $C_2$). The two photoelements are connected by a galvanometer *G* in such a way that no current passes through it when the elements are equally illuminated. It can easily be shown that the galvanometer deflection is proportional to the change in refractive index in the prism. The galvanometer is illuminated by the lamp $L_3$, and the light beam is reflected from the galvanometer mirror and the mirror *M* and makes a light spot on the photographic

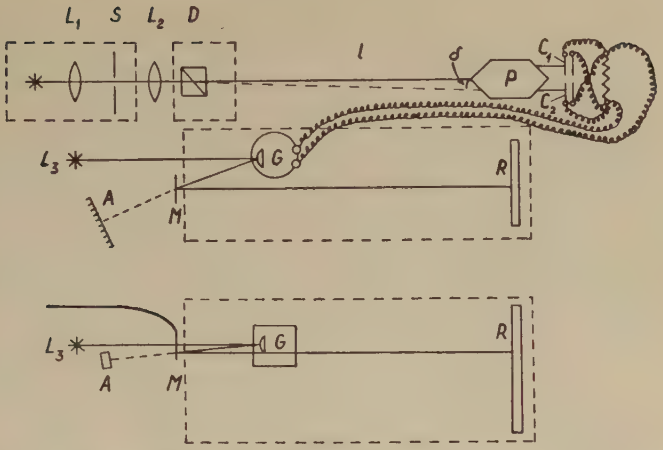

FIGURE 11. Sketch of the self-recording apparatus for adsorption analysis of solutions.

paper, *R*. When the refractive index changes, the light spot is consequently moved horizontally.

The volume of solution which has passed the cell is collected in a small flask hanging on a straight spring balance. The mirror *M* is attached to this spring and, consequently, the light spot moves vertically when the weight of the solution increases. It is thus seen that the refractive index of the solution and the weight of the solution which has passed the filter with adsorbent are recorded at right angles to each other on the photographic paper, *R*. In this way, the desired diagram is obtained. The whole apparatus is enclosed in a lightproof casing and is shown in FIGURE 12. The sensitivity is about $2.10^{-5}$ in refrac-

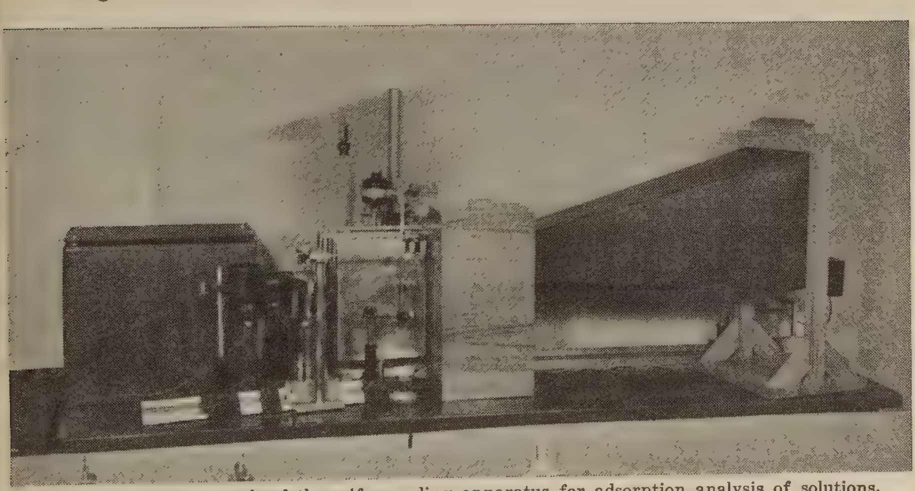

FIGURE 12. Photograph of the self-recording apparatus for adsorption analysis of solutions.

tive index, and, by using different springs, the apparatus can be used for volumes ranging from 10 to 1000 ml. The size of the photographic paper is about 20 by 30 cm.

Plate 1a shows a typical curve obtained with this apparatus.

**Apparatus for Gases and Vapors.** The apparatus used for adsorption analysis of gases and vapors is shown in figure 13. The mixture to be analyzed is mixed with a large amount of nitrogen ("the solvent") and introduced into the gas holder *A* through one of the glass tubes 25 or 26. The gas holder has been constructed according to Küster[22] and the compensating device 3-9 has the effect that the gas delivered always has the same pressure. From the gas holder, the gas passes the flowmeter *C*, the filter *D*, a cooling spiral immersed in water of 20°C, and the meter *F*, where the concentration is measured by observing the changes in thermal conductivity. *H* is a gas density balance, which can be used for special purposes. The meter *F* for the thermal conductivity contains four straight platinum wires mounted in four holes in a block of brass. The gas to be analyzed passes two of the wires, the other two being sur-

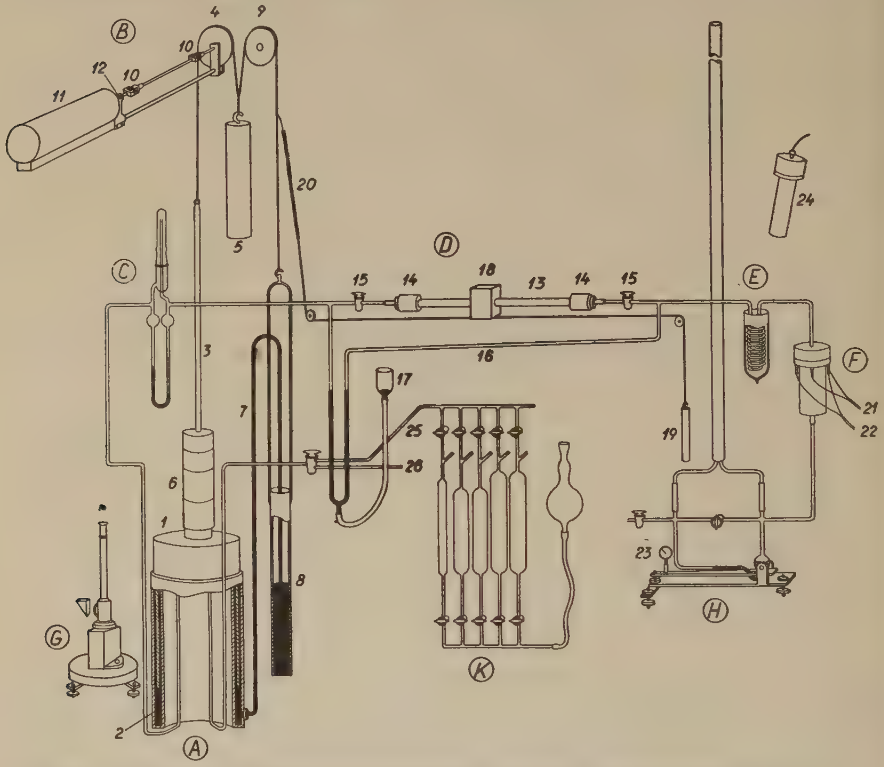

Figure 13. Sketch of the self-recording apparatus for adsorption analysis of gases and vapors.

rounded by a reference gas (*e.g.,* nitrogen). The four wires are heated electrically to about 125°C. and form the four resistances in a Wheatstone bridge. When the thermal conductivity of the flowing gas changes, the resistance of two of the wires changes, and the galvanometer *G* which is connected to the bridge, makes a deflection. This deflection is recorded on the drum 11, which is covered with photographic paper. It is seen from FIGURE 13 that the movement of the photographic paper is proportional to the movement of the gas holder and, consequently, proportional to the amount of gas that has passed the filter with adsorbent. The curve obtained thus has the desired shape with the concentration plotted against the volume.

PLATE 1b shows some typical curves obtained with this apparatus.

## SOME EXPERIMENTAL RESULTS

The method for adsorption analysis described here has been applied to a large number of different types of substances by Tiselius and his co-workers.[1–20] Only a brief summary of these results can be given here, and the reader is referred to the original papers for more detailed information.

Some homologous series (fatty acids, ethyl esters of fatty acids, alcohols, dibasic acids) have been studied rather extensively by Claesson.[18] In this case, it was found that displacement development was not applicable and frontal analysis had to be used. With activated carbon as adsorbent and ethyl alcohol as solvent, good separation was obtained. It was also found that the theory developed for frontal analysis gave rather good results. The calculations were much simplified, as Traube's rule (EQUATION 7) was valid with good accuracy. This is seen from FIGURE 14 where $^{10}\log k$ has been plotted against the number, $n$, of carbon atoms in the fatty acids for some ethyl esters of fatty acids. A typical example of a frontal analysis of fatty acids has already been given in FIGURE 10. It is also seen there that the retention volumes for the first three acids (with 8, 9, and 10 carbon atoms) are more closely together than for the three following (with 12, 14, and 16 carbon atoms). This is in accordance with EQUATION 8. Some examples of quantitative analysis are given in TABLE 1, where the composition has been calculated by means of EQUATION 6.

It has also been found that branched and unsaturated fatty acids are adsorbed less than the corresponding normal saturated fatty acids when carbon is used as adsorbent. When silica is used as adsorbent and the solvent is a non-polar liquid, all the normal saturated fatty acids are adsorbed to almost the same degree, the branched acids are adsorbed less, and the unsaturated more. In that case, it is, consequently, possible to separate the fatty acid mixture into these three groups, and it has also

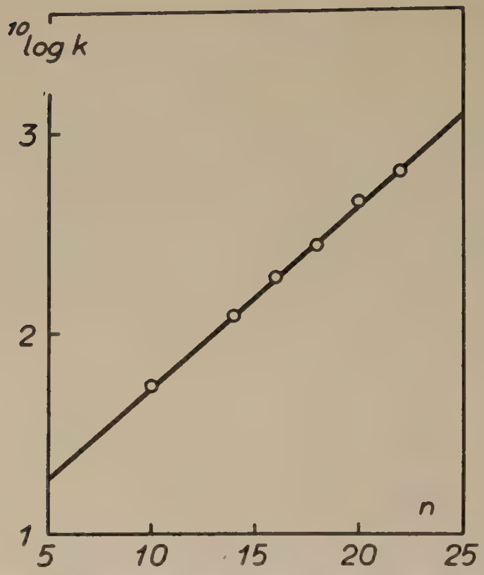

FIGURE 14. Traube's rule for ethyl esters of fatty acids.

been found that this can be done by means of displacement development (Claesson[19]). This effect is certainly due to the fact that the carboxyl group determines the adsorption on silica from non-polar solvents but not from polar solvents on activated carbon.

In some cases, it has been possible to use displacement development with extremely good results. One of the best examples of this is Tiselius' work on oligosaccharides (Tiselius and Hahn,[10] Weibull and Tiselius[15]). There, it was shown that a qualitative and quantitative analysis of mix-

---

PLATE 1

**a.** Frontal analysis of a mixture of 0.75% lauric and 0.75% palmitic acid in ethanol. Filter: 5000π "E-kol".

**b.** Displacement development of pure hydrocarbons. Developer: 1.5g. ethyl acetate. Adsorbent: 6g. carboraffin.

First row: 0.100 and 0.151g. *n*-pentane.
Second row: 0.151 and 0.200g. *n*-hexane.
Third row: 0.302 and 0.397g. *n*-heptane.

**c.** Displacement development of hydrocarbons. Developer: 1.5g. ethyl acetate. Adsorbent: 6g. carboraffin. Displaced substance:

0.137g. hexane + 0.143 heptane, 0.055g. hexane + 0.235 heptane, 0.018g. pentane + 0.104 hexane + 0.194g. heptane, 0.123g. pentane + 0.126g. hexane + 0.157g. heptane.

**d.** Frontal analysis of $CCl_2F_2$ containing a small amount of $CClF_3$.

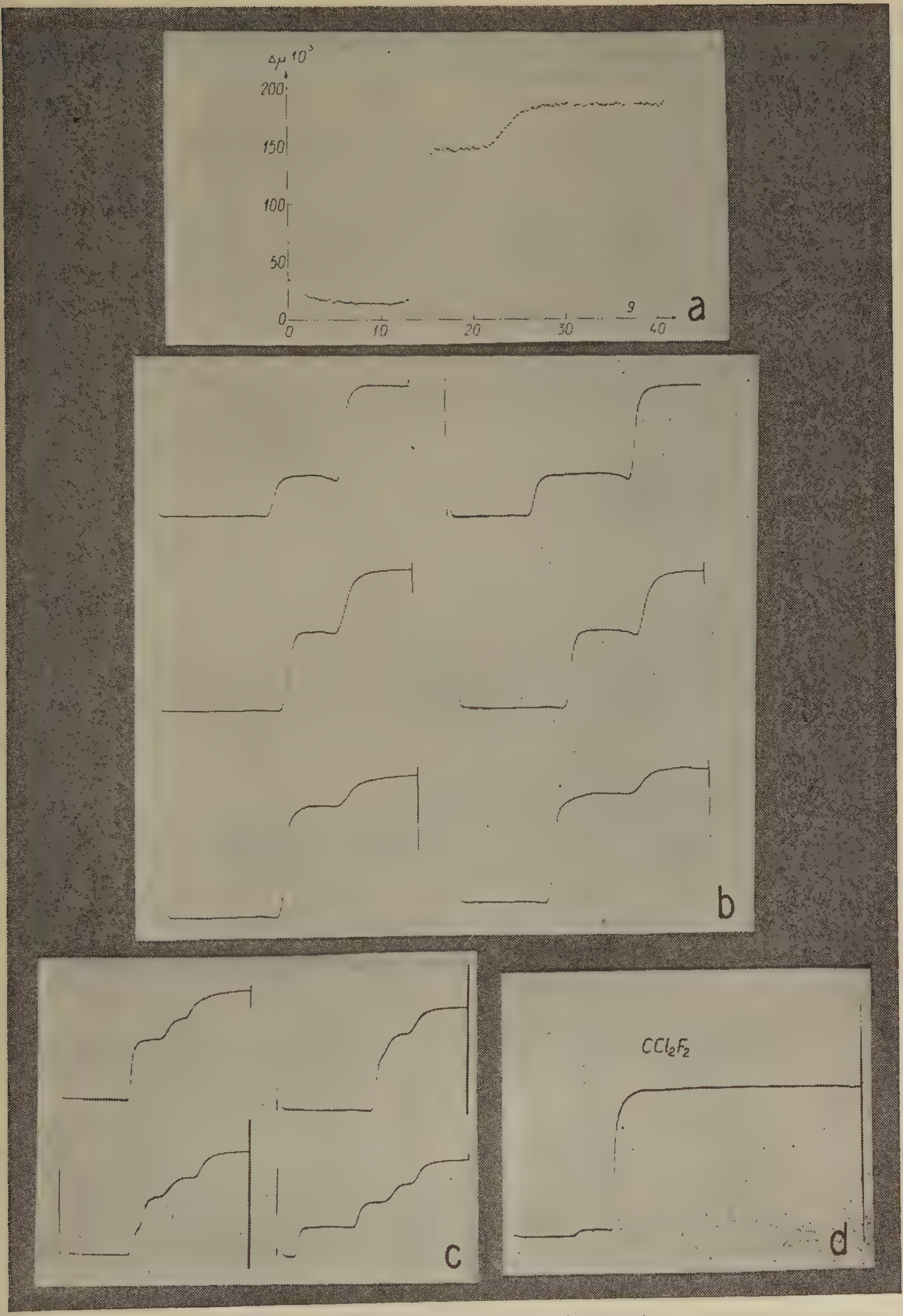

Plate 1 (*see caption on opposite page*).

tures containing mono-, di-, tri-, tetra-, penta-, and hexasaccharides was obtained from the heights and lengths of the steps in the displacement development diagram. Values obtained by Tiselius for the retention volumes of some typical saccharides are given in TABLE 2.

TABLE 1

FRONTAL ANALYSES OF MIXTURES

| Substances | Composition of the mixture (%) | |
|---|---|---|
| | Found value | Correct value |
| Caprylic acid, $C_7H_{15}COOH$ | 21 | 20 |
| Capric acid, $C_9H_{19}COOH$ | 19 | 20 |
| Myristic acid, $C_{13}H_{27}COOH$ | 20 | 20 |
| Palmitic acid, $C_{15}H_{31}COOH$ | 41 | 40 |
| Caprylic acid, $C_7H_{15}COOH$ | 14 | 10 |
| Pelargonic acid, $C_8H_{17}COOH$ | 10 | 10 |
| Capric acid, $C_9H_{19}COOH$ | 8 | 10 |
| Lauric acid, $C_{11}H_{23}COOH$ | 22 | 20 |
| Myristic acid, $C_{13}H_{27}COOH$ | 20 | 20 |
| Palmitic acid, $C_{15}H_{31}COOH$ | 27 | 30 |
| Adipic acid, $COOH(CH_2)_4COOH$ | 5 | 8 |
| Suberic acid, $COOH(CH_2)_6COOH$ | 16 | 15 |
| Sebacic acid, $COOH(CH_2)_8COOH$ | 31 | 31 |
| Dodecanedioic acid, $COOH(CH_2)_{10}COOH$ | 48 | 46 |

TABLE 2

SPECIFIC RETENTION VOLUMES FOR SACCHARIDES
0.5 per cent solution in water, adsorbent Norit P3.

| Substances | ml./g. |
|---|---|
| Arabinose | 9.9 |
| Xylose | 14.8 |
| Rhamnose | 9.5 |
| Levulose | 13.6 |
| Glucose | 16.0 |
| Galactose | 17.3 |
| Mannose | 18.5 |
| Saccharose | 43.5 |
| Lactose | 51.0 |
| Maltose | 60.0 |
| Raffinose | 68.0 |

Tiselius[12, 17] has also described some preliminary experiments with amino acids and peptides.

When the self-recording apparatus is used for experiments with large amounts of substance, it is also necessary to use large filters. It is, then, often difficult to obtain sharp boundaries. The shape of the boundaries is very much improved if smaller filters are mounted below the larger filters, with a small chamber (ca. 2 mm. in height and of the same diameter as the larger filter) between the filters. In this way, a very marked sharpening of the fronts is obtained. Some experiments of this type are shown in FIGURE 15. The filters used there were 20000π, 5000π, and 1250π, mounted above each other. As the experiments were carried out by means of displacement development, the different components are obtained in a pure state. It is also seen, from the figure, that the heights of the steps are constant and the lengths of the steps are proportional to the amount of substance. The values for the amounts obtained from the diagrams agree within 3 per cent with the correct values. It is consequently possible to prepare substances in pure state, in that way, in quantities which amount to several grams.

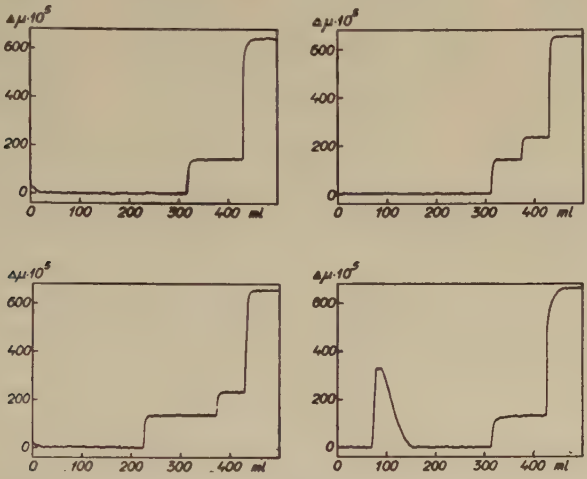

FIGURE 15. Displacement development of oligosaccharides. Developer: 4% phenol in water. Filter: 40000π + 5000π + 1250π carboraffin C. Displaced substance: 1.0 g. sucrose, 0.7 g. + 1.4 g. raffinose, 1.4 g. sucrose + 1.4 g. raffinose, 1.0 g. glucose + 1.0 = g. raffinose.

The apparatus for adsorption analysis of gases and vapors has until now mainly been used for analysis of hydrocarbons. It was found that displacement development worked well with these substances, and that esters of fatty acids were good developers. In PLATE 1b some experiments were shown, and in PLATE 1c it is seen that the same specific heights appear in the case of mixtures. From the great difference in specific height for pentane, hexane, and heptane, it is seen that the method is very selective and closely related compounds may be separated in this way. Some typical analyses of this kind are given in TABLE 3. It is seen that the accuracy of the quantitative analysis is good and the substances are well characterized by the heights of the steps. This height is given

TABLE 3

DISPLACEMENT DEVELOPMENT OF MIXTURES OF HYDROCARBONS
Developer: 1.5 g ethyl acetate; adsorbent: 6 g carboraffin

| Substances | Height of step | Composition of the mixture (%) | |
|---|---|---|---|
| | | Found value | Correct value |
| *n*-hexane<br>*n*-heptane | 56<br>78 | 20.4<br>79.6 | 19.7<br>80.3 |
| *n*-hexane<br>*n*-heptane | 56<br>79 | 48.4<br>51.6 | 48.2<br>51.8 |
| 2-methylhexane<br>*n*-heptane | 65<br>77 | 40.0<br>60.0 | 40.7<br>59.3 |
| *n*-pentane<br>*n*-hexane<br>*n*-heptane | 32<br>57<br>80 | 5.6<br>33.0<br>61.4 | 5.7<br>32.8<br>61.5 |
| *n*-pentane<br>*n*-hexane<br>*n*-heptane | 30<br>57<br>81 | 30.2<br>31.1<br>38.7 | 30.1<br>31.9<br>38.0 |
| *n*-hexane<br>Benzene | 56<br>89 | 43.1<br>56.9 | 43.2<br>56.8 |
| Cyclohexane<br>Benzene | 67<br>97 | 46.4<br>53.6 | 46.6<br>53.4 |

as a percentage of the height of the developer, and this constant thus becomes independent of the special properties of the thermal conductivity meter.

Frontal analysis can, of course, also be used for analysis of gases. This is seen from PLATE 1d, which shows a frontal analysis diagram of $C\,Cl_2F_2$ containing a small amount of $C\,ClF_3$.

## SUMMARY

In ordinary chromatographic analysis, colored bands are observed in a column filled with a suitable adsorbent. In the method described here, the concentration of the solution leaving the column is determined continuously in a small cell. This implies two fundamental advantages over the older chromatographic method. The procedure is especially suited for analysis of colorless solutions and independently of the color of the adsorbent, and, furthermore, the separation of the different components is greater in the percolate than in the column, which means increased selectivity. The theory for the method is first briefly discussed and, thereafter, the experimental arrangement is described. Two pieces of apparatus are described which are used for analysis of solutions, one being an interferometer, the other a self-recording apparatus. A self-

recording apparatus for adsorption analysis of gases and vapors is also described. Some typical examples of analyses carried out with this method are given in the last part of the paper.

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# SOME EXPERIMENTS IN SYSTEMATIC QUANTITATIVE CHROMATOGRAPHY

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During the past several years, our studies of the chemistry of propellants and explosives, under O.S.R.D. Contract OEMsr-881, have posed problems of the separation and quantitative determination of the substances in mixtures which contained from two to ten closely related or widely dissimilar organic compounds. Since any attempt to solve these problems by other than chromatographic methods would have been very difficult, we have consequently used chromatography for the quantitative determination of the compounds in these complex mixtures. I do not propose to describe the analysis of propellants and explosives as such, but rather to discuss the problems which arise in the adaptation of the usual chromatographic procedure to the analysis of mixtures of organic compounds with a precision comparable to that which is ordinarily obtained in inorganic analysis. These problems will be illustrated with data obtained at the California Institute of Technology by a group of about a dozen workers, to whom I wish to make full acknowledgment for the data which I shall present.

The materials which were analyzed in our studies of propellants and explosives may conveniently be classified in three categories: (1) mixtures of known qualitative composition; (2) mixtures whose composition could at least to some extent be surmised from their sources; and (3) mixtures of completely unknown composition. As an example of the first class, we may cite the quantitative determination of diphenylamine and diethyl phthalate in an American powder which contained, in addition, nitroglycerin and nitrocellulose. The second type of material is illustrated by various aged samples of this same powder, in which we wished to determine the fate of the stabilizer, diphenylamine. From information in the technical literature[1, 2] and from the chemical properties of diphenylamine, it was clear that provision must be made for the estimation of approximately twenty nitro and nitroso derivatives. Finally, explosives and powders of completely unknown composition were frequently encountered, and it was then often necessary to isolate and identify compounds which had not been studied previously, before a method for the complete quantitative analysis of the material could be devised.

Let us, first, turn our attention to the materials and methods which were used: the adsorbent, the developers, the elution, the determination of the compound in the eluate by spectrophotometry, as well as certain anomalous behaviors which were observed; and then let us consider the results of the application of chromatography to the quantitative analysis of the three types of materials which have been described.

All of the techniques which I shall discuss are based upon the use of silicic acid as an adsorbent. Silicious adsorbents have been used in a number of chromatographic studies.[3-11] Reagent silicic acid produced by several manufacturers has properties which recommend it as an adsorbent: it is pure white and thus permits ready detection of faintly colored zones; it is inert; it is finely powdered; and it may be modified in activity by appropriate treatment and yet, as received from the manufacturer, it does not show important variations from lot to lot. However, because of the slow filtration of solvents through the finely powdered adsorbent, it is advantageous to use a mixture of two parts by weight of silicic acid and one part of Celite 535.

For quantitative chromatography, it is important that adsorbent and solvents do not contribute impurities. Solvents may be purified by simple distillation in all-glass apparatus, and the adsorbent may be purified by pre-washing with redistilled solvents immediately before the chromatogram is run. Pre-washing of the adsorbent, however, has effects other than mere purification. These are illustrated in FIGURE 1. The variant in these experiments was the type of pre-wash, whereas the size of the column (19 x 150 mm.), the amount of 4-nitrodiphenylamine (0.5 mg.), and the type of developer (1:1 benzene-ligroin) remained constant. I shall speak of solvents in terms of the ratios of the volumes of the constituents and of the quantity of solvents in terms of the convenient convention "V ml." which we shall define as the volume of solvent which is required to wet completely a column of adsorbent. The alcohol pre-wash used V ml. of absolute alcohol and 2 V ml. of ligroin, the ether pre-wash

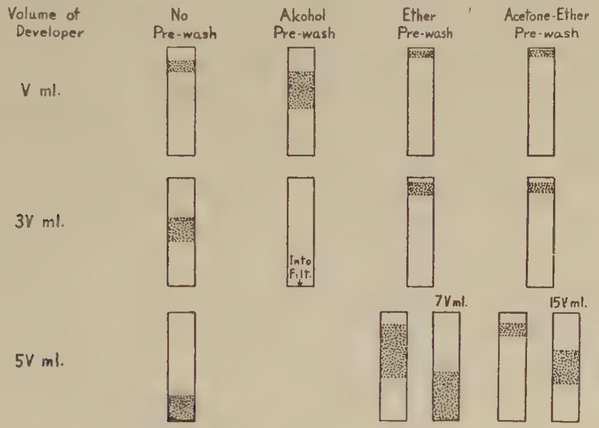

FIGURE 1. Effect of type of pre-wash upon the rate of development and width of a zone of 4-nitrodiphenylamine.

V ml. of ether and 2 V ml. of ligroin, and the acetone-ether pre-wash 0.2 V ml. of ether, V ml. of 1:1 acetone-ether, 0.8 V ml. of ether, and V ml. of ligroin. The zones as shown are idealized; the distribution of the compound was not uniform and the edges were not sharp.

A comparison of the position of the zone after equivalent development shows that the strength of the adsorbent increases in the following order: alcohol pre-washed adsorbent <unpre-washed adsorbent <ether pre-washed adsorbent <acetone-ether pre-washed adsorbent. Only the ether and acetone-ether pre-washes have been used in practice, and several differences may be noted in the behavior of the zone after these pre-washes. On ether pre-washed adsorbent, the zone moves with increasing speed and width in the lower sections of the column, whereas on acetone-ether pre-washed material, the rate of movement is regular and the increase in width, although apparent, is much less.

The explanation of the effect of the pre-wash is probably connected with the water content of the adsorbent, since, as is well known, the adsorptive strength of silica gel, of which silicic acid is surely a form, is a function of the water content and increases as the water content decreases until this content is decreased to about 5%.[12, 13] It has been found that, at 200° C., silicic acid loses weight for about 40 minutes, and that this adsorbent then behaves as though it had been pre-washed with acetone-ether. Another experiment showed that the loss in weight, on heating, is equivalent to the weight of the water which may be found in the solvents after pre-washing with acetone-ether. Apparently, then, the anhydrous solvents of the pre-wash activate the adsorbent by removing water. Ether, because it dissolves water less readily then acetone-ether, removes water less completely and less uniformly, with the consequent result that there is a gradation of the properties of the adsorbent from the top to the bottom of the column and, thus, the zones behave as indicated. Acetone-ether, however, produces a more uniform column, and the behavior of zones is more uniform as well. The de-activating effect of alcohol may be explained if we assume that alcohol, because of its structural similarity to water, merely replaces water in the adsorbent.

The adsorption affinity of many organic compounds on silicic acid is such that benzene or benzene-ligroin moves the zones at a reasonable rate but may not produce entirely satisfactory zones or separations. The pronounced effect of the developer upon the zone may be seen in FIGURE 2. The adsorbent was ether pre-washed, the only variant being the type of developer. The rates of development are similar, but the ether-ligroin produces a much more regular rate of movement and a more compact zone than does benzene-ligroin. Ethyl acetate or acetone in ligroin behaves very nearly as ether-ligroin.

Ligroin or benzene solutions of ether, ethyl acetate, or acetone, not only usually produce well-defined zones, but they often produce clean-cut separations of mixtures which benzene or benzene-ligroin can separate

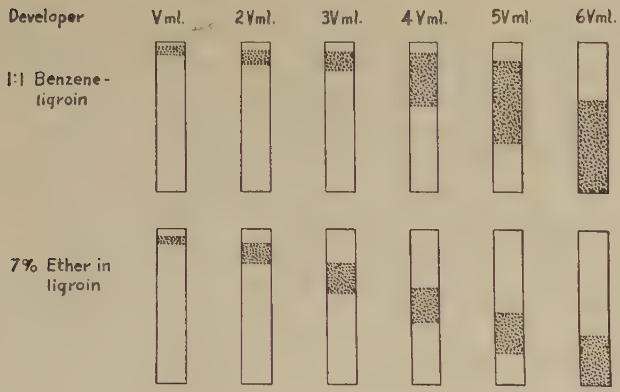

FIGURE 2. Effect of type of developer on the formation of a zone of 4-nitrodiphenylamine.

poorly if at all. However, developers which contain alcohol rarely are satisfactory. Sometimes, even ternary mixtures of benzene and ligroin with ether, ethyl acetate, or acetone are helpful. Three isomeric dinitrodiphenylamines may be used as an excellent illustration of the ease with which one developer will separate two compounds which cannot be separated by another. The relations may be seen in FIGURE 3. Thus,

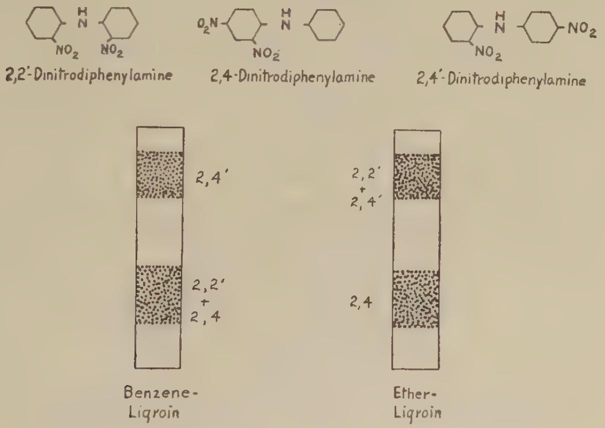

FIGURE 3. Separation of isomeric dinitrodiphenylamines.

benzene-ligroin readily separates 2,4′-dinitrodiphenylamine from the mixture of 2,2′- and 2,4-dinitrodiphenylamine, whereas ether-ligroin parts 2,4-dinitrodiphenylamine from the mixture of 2,2′- and 2,4′-dinitrodiphenylamine. The separation of the three compounds is, of course, relatively simple, since the mixture which may be isolated from one chromatogram may readily be separated by re-chromatographing with different development.

Common eluents such as alcohol, ether, and mixtures of alcohol or acetone in ether elute most compounds from silicic acid without difficulty. Indeed, only those compounds with an N-nitroso linkage required care in order to prevent decomposition. TABLE 1 shows typical results which may be obtained when separately weighed portions of a compound are chromatographed and eluted and the quantity is determined spectrophotometrically. Since the N,N-diphenylurea is so strongly adsorbed that ether itself is a good developer, it was necessary to use ethanol in ether as an eluent. It is seen that a 5 per cent solution was as effective an eluent as a 10 per cent solution. Usually, the calculated recovery will be 98 to 101 per cent of the weighed amount which was placed on the column. This precision is of the order which one might expect from a consideration of the errors involved.

TABLE 1

ELUTION OF N,N-DIPHENYLUREA FROM THE CHROMATOGRAPHIC COLUMN

| Eluent % EtOH in $Et_2O$ | Initial weight mg. | Weight recovered mg. | Per cent recovered |
|---|---|---|---|
| 5 | 6.23 | 6.15 | 98.7 |
| 7.5 | 6.23 | 6.17 | 99.0 |
| 10 | 6.23 | 6.16 | 98.9 |
| 10 | 6.23 | 6.15 | 98.7 |
| 5 | 9.44 | 9.46 | 100.2 |
| 5 | 9.44 | 9.47 | 100.3 |
| 5 | 7.77 | 7.75 | 99.6 |

Since the spectrophotometer is so valuable in determining the quantities of 1 to 10 mg. which are most conveniently isolated, a few remarks about corrections in spectrophotometry should be made. Two familiar corrections are required in any optical instrument such as the Beckman Quartz Photoelectric Spectrophotometer which uses two optical cells, one for solvent and the other for solution. These corrections are necessary because few cells are exactly 1.000 cm. in thickness, and two cells are seldom so matched or so clean that one has no extinction relative to the other. Another correction which must be applied to spectrophotometric reading is one which corrects for small amounts of impurities which are not removed by pre-washing or distillation of solvents. This is estimated by means of a blank determination. The sum of the corrections rarely exceeds ± 1 or 2 per cent of the extinction. In an instrument of this type, the spectra of most compounds which contain benzene nuclei can be satisfactorily determined if the concentration is from 0.5 to 5 mg. per 100 ml., depending upon the intensity of absorption.

For definitive results by spectrophotometric methods, the solvent must be specified and, consequently, the eluent must often be replaced by a suitable solvent. Alcohol is an excellent solvent because its transparency permits readings at rather short wavelengths. However, it is in this transfer from one solvent to another that the greatest errors in a quantitative chromatographic determination may easily be introduced, since so much eluent must be removed from so little compound. The usual method of evaporating the solvent under reduced pressure is of course satisfactory if the eluted compound is non-volatile, but this is a very strict requirement, since the loss of 0.1 mg. would cause an appreciable error. On the other hand, solvent may often be removed from a volatile compound by allowing the solvent to evaporate from the zone after it has been cut from the column and then eluting the dry adsorbent directly with alcohol. Even a compound such as N-nitroso-N-ethylaniline, which is volatile and decomposes on prolonged contact with adsorbent, may be determined with reasonable accuracy if 100 mg. of a high-boiling compound such as diethylene glycol, which does not interfere with spectrophotometry, is added to the ether eluate before evaporation is begun. Thus, when the ether has evaporated, the 1 to 2 mg. of compound is dissolved in diethylene glycol and its vapor pressure correspondingly diminished. Actually, the recovery is only about 90 per cent, but without the high-boiling liquid it is about 10 per cent. It may even be that the solvents with which the column is wet and which, of course, are carried into the eluate by the eluent, do not interfere with spectrophotometry. For example, ligroin of boiling range 60-70° contains appre-

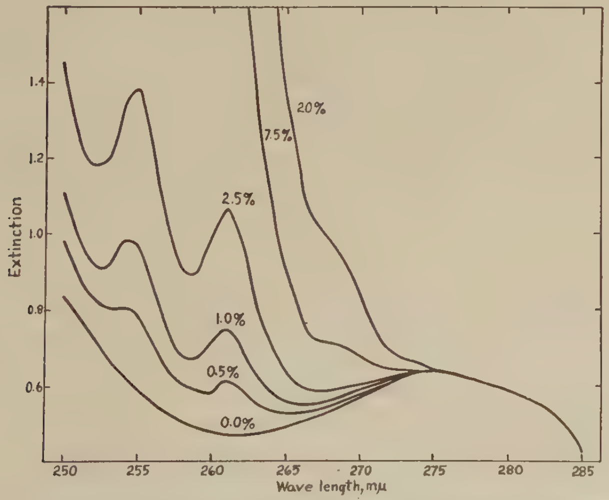

FIGURE 4. Extinctions of solutions containing the same concentration of diethyl phthalate and varying percentages of ligroin.

ciable amounts of benzene and, yet, 20 to 30 per cent of this solvent may be present in alcohol before the extinction differs appreciably from that of pure alcohol at wavelengths longer than 280 mμ. This effect is illustrated in FIGURE 4. The maxima at 255 and 262 mμ are caused by the presence of benzene. However, the benzene and ligroin have caused no increase in extinction at 275 mμ, the maximum of diethyl phthalate which is used for quantitative determinations. Thus, if the spectrophotometric constants of a compound are unaltered by the presence of the ligroin, measurement may be made directly on the alcohol eluate at wavelengths longer than 275 to 280 mμ, but at shorter wavelengths all extraneous solvents must be removed.

I should like to discuss, briefly, two anomalous behaviors which many compounds showed on silicic acid; they are inversions and what may be called "double zoning."

When one considers the number of chromatographic investigations which have been published in the last decade, comparatively few examples[6, 14-18] of inversion in the relative positions of two compounds by change of adsorbent or developer may be cited. On silicic acid, we have found that inversions by change of developer occur very frequently. A few examples which we have observed are shown in FIGURE 5. When a nitrate ester is one of a pair, an inversion is very likely to occur, because nitrate esters develop rather readily with benzene-ligroin mixtures and very much more slowly with ether-ligroin mixtures than would be expected, while most other compounds behave as one would anticipate. The examples in this figure, as well as others, have indicated that inverted compounds usually are rather dissimilar in structure. If we consider the dinitrodiphenylamines again (FIGURE 3), it is evident that, if the degree of change had been greater, an inversion would have occurred. Consequently, the ability or inability of one or another developer to separate

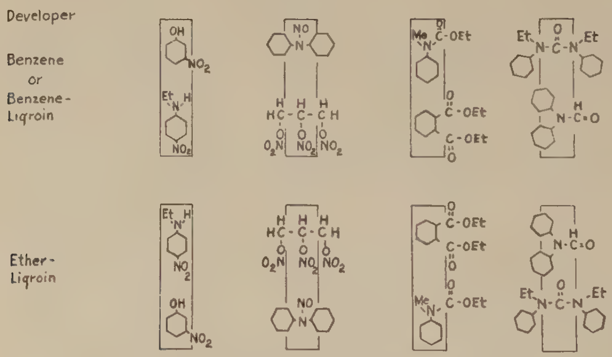

FIGURE 5. Inversion in relative position by changing developer.

two compounds, as well as inversions by change of developer, are merely degrees of the same effect. Some inversions, for example, that of N-nitrosodiphenylamine and nitroglycerin, have been used to advantage in devising separations. Thus, the separation of 2 mg. of N-nitrosodiphenylamine from 100 to 200 mg. of nitroglycerin with benzene-ligroin was not clean-cut because of the disproportionate amounts. Yet, when ether-ligroin was used, the nitroglycerin remained strongly adsorbed, while the N-nitrosodiphenylamine developed rapidly and separated easily. Although these instances of inversion are the result of rather extreme changes in the type of developer, examples might be cited of inversions which are caused merely by altering the proportions of the solvents in a developer. For example, N-ethylcarbanilide is more strongly adsorbed when a 2 per cent solution of ethyl acetate in ligroin is used, whereas 4-nitro-N-ethylaniline is more strongly adsorbed when the concentration is 5 per cent of ethyl acetate.

Of the approximately 100 compounds which we have chromatographed in studies of powder and explosives, a relatively large number exhibit the undesirable effect which may be termed "double zoning," that is, when a presumably homogeneous compound is chromatographed, it may, under certain conditions, produce two well-separated zones which cannot be distinguished by other than chromatographic methods, and each of which, when eluted and re-chromatographed, again produces two zones. Nearly always, the upper zone is the smaller of the two. Double zoning has been observed with such structurally different compounds as 4-nitroso-N,N-diethylaniline, N,N-diphenylurethane, carbanilide, and various nitro derivatives of diphenylamine. Although reasonable explanations of the cause may sometimes be given, no one explanation will suffice for all examples. In some instances, the presence of benzene in the developer seems to produce double zones which may, however, be prevented by changing the ratio of benzene to the other solvent in the developer; in other instances, precipitation of the compound by a succeeding developer in which it is insoluble may be the important factor. Double zoning appears to be similar to the blurred separation of the carotenoid methylbixin as observed by Zechmeister and Escue.[19]

Having described the methods and materials which were used in quantitative analyses, let us now consider the application of these methods to the analysis of the three types of material which have been mentioned.

The procedures which were required for the analysis of the powder of qualitatively known composition necessitated the separation of diphenylamine and diethyl phthalate not only from each other but also from nitroglycerin and nitrocellulose. Since diphenylamine, diethyl phthalate, and nitroglycerin may readily be separated from nitrocellulose by extracting the finely divided powder for several hours with methylene chloride in a Soxhlet apparatus, it is necessary to provide only for the chromatographic separation of diphenylamine, diethyl phthalate, and nitroglycerin in an extract of the powder. A study of the chromatographic properties of these compounds, individually and in mixture, showed that diethyl phthalate is considerably more strongly adsorbed than diphenylamine and nitroglycerin, which may easily be washed from the column, while diethyl phthalate remains strongly adsorbed. Diphenylamine, on the other hand, is the least strongly adsorbed and may be developed into the middle or lower portion of the column by developers which do not move diethyl phthalate and nitroglycerin from the top of the column. Thus, the actual chromatographic separation of the compounds presents no difficulties.

In addition to determining the method of separation, it was also necessary to consider the quantities which must be isolated. Since spectrophotometry was to be used, the spectrophotometric constants of the compounds were determined. The results showed that, if one milligram of diphenylamine was isolated, a dilution to 200 ml. with ethanol would give an appropriate value of the extinction, but that 15 to 20 mg. of diethyl phthalate would be required before the extinction would be sufficiently high at a satisfactory dilution. Since the approximate amount of diphenylamine in the powder was 0.7 per cent and that of diethyl phthalate 3.3 per cent, it may be calculated that 150-mg. and 500-mg. samples would be satisfactory for the respective analyses.

On the basis of this information, the following procedures were devised, as may be seen from FIGURE 6. About 2.5 g. of the powder were extracted

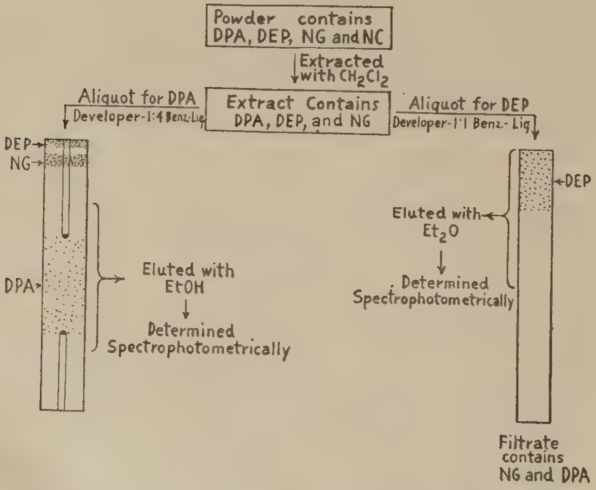
Powder contains DPA, DEP, NG and NC
Extracted with $CH_2Cl_2$
Aliquot for DPA
Developer-1:4 Benz.-Lig
Extract Contains DPA, DEP, and NG
Aliquot for DEP
Developer-1:1 Benz.-Lig
DEP
NG
DPA
Eluted with EtOH
Determined Spectrophotometrically
DEP
Eluted with $Et_2O$
Determined Spectrophotometrically
Filtrate contains NG and DPA

FIGURE 6. Determination of diphenylamine and diethyl phthalate.

with methylene chloride, which is equivalent to benzene as a developer. The extract in about 50 ml. of methylene chloride was then diluted to 100 ml. with ligroin, and 5-ml. aliquot portions were taken for the analysis for diphenylamine and 20-ml. aliquot portions for diethyl phthalate. The aliquot portions for diphenylamine were diluted with ligroin, so that the solvent was 1:4 methylene chloride-ligroin, whereas the 1:1 methylene chloride-ligroin was a suitable solvent for diethyl phthalate.

The diphenylamine was determined by chromatographing on a 25 x 150 mm. column of a mixture of 4:1 silicic acid-Celite which had been pre-washed with ether and ligroin. The diphenylamine was developed into the middle of the column with 1:4 benzene-ligroin and a post-wash, while the nitroglycerin and diethyl phthalate remained at the top. All of these compounds are colorless. Consequently, the zone of diphenylamine was located by streaking with a solution of sodium nitrite in sulfuric acid, which produces a blue color at the position of the zone, and the zone of nitroglycerin by streaking with diphenylamine in sulfuric acid, which also produces a blue color. Diethyl phthalate is more strongly adsorbed than nitroglycerin. In order to prevent the inadmissible loss which would result from the removal of a streak which had passed through a zone, the following method was adopted. The column was streaked carefully upward from the bottom until the production of color indicated the bottom of the zone and, likewise, from the top until the upper boundary had been found. After the zone had thus been delimited, the removal of the streak before elution resulted in inappreciable loss of compound. This particular streak reagent, sodium nitrite in sulfuric acid, in common with numerous other reagents, is affected by the solvents with which the column is wet. Thus, on clean adsorbent which is wet with benzene, an orange-brown color is produced, while on adsorbent which is wet with ligroin of boiling range 28-38° the streak is colorless. Consequently, after development of the chromatogram, it is well to post-wash with ligroin, in order to obtain the maximum contrast between the normal color of the reagent on free adsorbent and that color which is produced as the zone is reached. The zone was then eluted directly with alcohol and the quantity was determined spectrophotometrically. Diphenylamine is too volatile to permit evaporation of ether from an eluate, although the solvent may be permitted to evaporate from the zone before elution. However, studies showed that the solvents with which the column is wet and which are removed by elution do not interfere with spectrophotometric determination.

The diethyl phthalate was isolated by chromatographing on a 19 x 150 mm. column of ether pre-washed adsorbent and washing nitroglycerin and diphenylamine into the filtrate with 1:1 benzene-ligroin. The upper half of the column was eluted with ether, the solvent was evaporated under reduced pressure, a few milliliters of redistilled alcohol were added and evaporated to remove traces of residual solvent, and the residue was taken up in alcohol and estimated spectrophotometrically. Since no streak reagent was available for diethyl phthalate, the column had to be cut arbitrarily. However, experiments showed that a generous margin of safety was present if the upper half of the column was eluted.

A long study of these procedures showed that, usually, the results from duplicate aliquot portions of the same extract agreed to within one per cent of the amount of the compound, and that the values from duplicate extracts of the same powder had a maximum spread of about 3 per cent of the compound present. Thus, if 3.30 per cent of diethyl phthalate were actually present, values might range from 3.25 to 3.35 per cent, or 0.1 per cent of the powder. Likewise, if 0.70 per cent of diphenylamine were present, the values might range from 0.69 to 0.71 per cent, or 0.02 per cent of the powder. These results are representative of those which we obtained with quantitative chromatographic-spectrophotometric procedures for other compounds.

I should mention that no chromatographic-spectrophotometric procedure for nitroglycerin was devised because the compound has no maximum in its spectrum at wavelengths between 220 m$\mu$ and the end of the visible range, and, furthermore, chemical means, such as reduction with ferrous chloride and subsequent titration with titanous chloride of the ferric ion which is formed, are satisfactory for its determination.

The actual study of the changes and reactions of the stabilizer, diphenylamine, in the aging powder was preceded by investigation of the chromatographic and spectrophotometric properties and methods for the separation of the approximately twenty anticipated derivatives. Subsequent application of the procedures to the various heated samples then gave the results which are shown in FIGURE 7. The thirteen compounds of which the increase and decrease are represented in this figure, as well as four others (2,4-dinitrodiphenylamine, N-nitroso-2-nitrodiphenylamine, N-nitroso-2,4′-dinitrodiphenylamine, and N-nitroso-4,4′-dinitrodiphenylamine) which were detected in only insignificant amount, are a complex system which includes nitroso, mononitro, nitrosonitro, dinitro, nitrosodinitro, trinitro, tetranitro, pentanitro, and hexanitro derivatives of diphenylamine. The decrease of diphenylamine is not shown in FIGURE 7. The original 7 mg. per g. decreased at an essentially constant rate and was exhausted in about 5 days. The diversity of structure has permitted a few generalizations about the effect of the number and position of the substituent groups upon the relative adsorption affinity of the compounds. Thus, diphenylamine itself is very weakly adsorbed, but 4-nitrodiphenylamine has a markedly increased adsorption affinity. On the other hand, 2-nitrodiphenylamine is only slightly more strongly adsorbed than diphenylamine. This effect of a nitro group in the 2 position is such that dinitro derivatives, with the exception of 4,4′-dinitrodiphenylamine, are all less strongly adsorbed than 4-nitrodiphenylamine, and the trinitro and tetranitro derivatives are less strongly adsorbed than 4,4′-dinitrodiphenylamine. Chelation of the 2-nitro group with the amino group may very well be the cause of this effect. More highly nitrated derivatives are very strongly adsorbed. An N-nitroso group may either increase or decrease the adsorption affinity: for example, N-nitrosodiphenylamine is more strongly adsorbed then diphenylamine, but N-nitroso-4-nitrodiphenylamine is less strongly adsorbed than 4-nitrodiphenylamine, although still more strongly adsorbed than N-nitrosodiphenylamine.

Because of the relationships in the adsorption affinities, the methods which were devised for separating this complex mixture were not unduly complicated. The weakly adsorbed compounds could be isolated easily by individual chromatograms. The more strongly adsorbed compounds could then be separated into several groups, and the compounds in the groups could be parted by re-chromatographing with an appropriately different developer. The most complex mixture was that in the 35-day sample which contained no less than 10 derivatives. Usually, the analyses were less difficult because of the smaller number of compounds which had to be isolated. In general, the complete analysis of one sample required 3 to 4 days and 10 to 12 chromatograms.

These methods were not as refined as those for diphenylamine and diethyl phthalate, but it is probable that these results indicate, to within 5 or 10 per cent, the content of the derivatives in the powder.

The derivatives which are shown in FIGURE 7 account for only 60 per cent of the original diphenylamine. The fate of the remainder is still unsolved and, in any event, is a problem beyond the scope of this discussion.

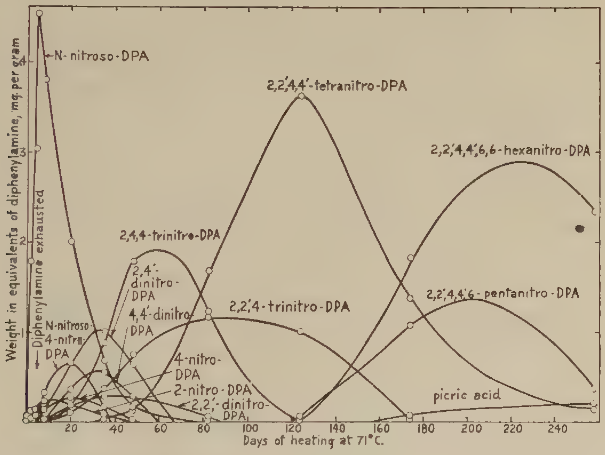

FIGURE 7. Derivatives formed from diphenylamine in double base powder.

Captured enemy munitions are perhaps the best examples of mixtures of unknown composition which it was our task to analyze. The general method of study, which is similar to the usual methods for, say, a natural extract, may be described, briefly, as follows. An extract of the powder was placed on a column and developed only slightly. Since the major constituents of most powders are colorless, it was necessary to detect the zones by application of various reagents which indicated whether a nitric ester such as nitroglycerin or diethylene glycol dinitrate as well as other compounds were present. The nitric ester was then tentatively identified by comparison of its chromatographic properties with those of known compounds. A few chromatograms with different developers usually indicated a method of separation for the other detected compounds, which were then shown by spectrophotometric examination to be known or unknown. Unknown compounds were isolated in sufficient quantity to permit identification by standard methods. Eluates of sections of the column which did not react with reagents were examined for the presence of undetected compounds. After the qualitative analysis was complete, a method for the quantitative analysis was devised and applied. In one instance, however, when the identification of one compound was difficult, its spectrophotometric properties were determined and the quantitative analysis was completed before the qualitative. TABLE 2 shows the results of the analysis of a German powder.

TABLE 2

COMPOSITION OF A GERMAN POWDER

| Compound | Percentage |
|---|---|
| N-Ethyl-N-phenylurethane | 1.58, 1.57 |
| N,N-Diphenylurethane | 1.75, 1.79 |
| N,N-Diphenylurea | 0.18, 0.19 |
| Diethylene glycol dinitrate | 32.7 |
| Nitrocellulose and inorganic compounds | 62.8 |
| Total | 99.0 |

The two results which are cited for the urethanes and diphenylurea were obtained from two separate extracts of the powder. Although the sum of the constituents is not 100 per cent, no evidence for the presence of other compounds was obtained, and there is every reason to believe that the discrepancy results from inaccuracies in the determination of diethylene glycol dinitrate and nitrocellulose, which are the major components and the most difficult to determine accurately. These were determined by standard methods, since no chromatographic-spectrophotometric method was devised for their determination.

The applications of quantitative chromatography which I have described have been in a specialized field, but I believe they indicate that, when relatively simple stable organic molecules are to be determined, complex mixtures of organic compounds can be analyzed with comparative ease.

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# FRACTIONATION AND ANALYSIS OF HYDROCARBONS BY ADSORPTION

*By* BEVERIDGE J. MAIR

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This report[1] reviews two methods for separating hydrocarbons by adsorption which were developed in connection with the work of the American Petroleum Institute Research Project 6 at the National Bureau of Standards. These adsorption processes have been used primarily to separate mixtures of aromatic, paraffin, and naphthene (or cycloparaffin) hydrocarbons, as in the gasoline and kerosene fractions of petroleum, into two portions, one containing only aromatic hydrocarbons, the other only paraffin and naphthene hydrocarbons. This resolution of the mixture into two portions simplifies the problem of analysis, since an analytical distillation of the separated portions may be used to obtain much more information than is obtainable from an analytical distillation of the original mixture.

**Description of the First Method.** With the first method, the mixture of hydrocarbons is introduced into the top of a column containing an excess of solid adsorbent (silica gel), and when it has completely entered the adsorbent a low boiling paraffin hydrocarbon, such as *n*-pentane, is added in sufficient quantity to remove from the column the paraffin and naphthene hydrocarbons but not the aromatic hydrocarbons. The latter are then removed by adding an appropriate desorbing liquid such as methanol. The paraffins, naphthenes, and pentane are thus obtained as a mixture from which the pentane is easily removed by distillation. The aromatic hydrocarbons are obtained as a mixture with pentane and methanol. The methanol is easily removed by extraction with water, and the pentane is removed by distillation. Refractive indices are determined on the filtrate fractions and are used to distinguish the aromatic portion from the paraffin-naphthene portion. Silica gel adsorbent of 28 to 200 mesh size, with a natural rate of flow without application of pressure, is normally used in this method.

This method was used on a comparatively large scale in a Pyrex glass column, 10 cm. in diameter and 300 cm. in length, holding approximately 15 kg. of silica gel. This column was capable of separating from 500 to 1000 ml. of aromatic material.

This method of separation was tested on a known mixture of 17 pure hydrocarbons, the normal boiling points of which covered a range from 60° to 174° C. The mixture included all of the 5 normal paraffins from *n*-hexane through *n*-decane, the isoparaffin 2-methylpentane, the 4 normal alkylcyclohexanes from cyclohexane through *n*-propylcyclohexane, and all of the 7 possible aromatic hydrocarbons from benzene through isopropylbenzene. The separation of the aromatic hydrocarbons from the paraffins and naphthenes was quantitative within the limits of measurement, and their recovery was complete within the normal operating loss of material in processing (see reference 1, first item).

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[1] Only a brief summary of this work is given here, since a detailed description is available in the following references:
Mair, B. J. & A. F. Forziati. J. Res. NBS 32: 165. 1944.
Mair, B. J. J. Res. NBS 34: 435. 1945.
Rossini, F. D., B. J. Mair, A. F. Forziati, A. G. Glasgow, Jr., & C. B. Willingham. Proc. Am. Petroleum Inst. 23 (3): 7. 1942; Oil Gas J. 41 (27): 106. 1942; Petroleum Refiner 21(11): 73. 1942.

**Description of the Second Method.** With the second method, the mixture of hydrocarbons is introduced into the top of a column of silica gel and, when the liquid level just reaches the top of the silica gel, a suitable desorbing liquid, such as ethyl or isopropyl alcohol, is added. The desorbing liquid forces the hydrocarbon portion down the column, during which passage the hydrocarbon portion is fractionated according to the adsorbability of the various components. The paraffin plus naphthene hydrocarbons issue from the column first, followed by the aromatic hydrocarbons and, finally, by alcohol.

On a small scale, this method is particularly suitable for the determination of aromatic content, the analysis being made by determining the fraction of the total volume constituted by the aromatic portion. Refractive indices are determined on the filtrate fractions, and are used to allocate, to the proper portions, the volumes of those fractions which occur at the "breaks" between the paraffin-naphthene and the aromatic portions and between the aromatic portion and alcohol.

The apparatus used for the analysis is made of Pyrex glass and consists essentially of a reservoir sealed to the column proper, which itself consists of two sections, an upper section, 2.2 cm. in diameter and 50 cm. in length, and a lower section, 1.0 cm. in diameter and 75 cm. in length. The column holds 160 g. of silica gel and will separate about 20 ml. of aromatic material. The silica gel is finer than that used with the first method and is of a nearly uniform particle size, about 60 per cent being between the 200 and 325 mesh sizes. Pressures of 5 to 8 lbs./sq. in., applied at the top of the reservoir from a source of inert gas under pressure, are used with material of the gasoline range, and somewhat higher pressures with material of the kerosene range. Usually, fractions of 5 or 10 ml. are collected, except in the neighborhood of the "breaks" between the portions, where 1-ml. fractions are taken.

Results of the analyses of synthetic mixtures with this method indicate that the aromatic content can be determined to within ±0.2 per cent.

The second method was used also for the separation, on a small scale, of monoolefin from paraffin and from aromatic hydrocarbons. At room temperature, considerable reaction of the olefins, as by polymerization, occurs. This polymerization is reduced but not entirely eliminated by sweeping the column with nitrogen prior to the introduction of the hydrocarbon, and by operating at lower temperatures.

Current work on this second method at the National Bureau of Standards includes the extension of the process to larger laboratory scale operations and the investigation of differences in the adsorbability of hydrocarbons of the same type due to differences in molecular size and structure.

# STEREOCHEMISTRY AND CHROMATOGRAPHY

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It is one of the oldest theses in chemistry that all properties of a given substance are functions of its chemical structure. This fundamental idea is the starting-point for manifold efforts, all of which have attempted to locate within the framework of the molecule those sections which are made responsible for certain physical or chemical properties. A detailed study of the functional groups should not, however, distract the attention of the organic chemist from such characteristics of the compound which, possibly, are caused by the general shape of the molecule. The classic field for such investigations is stereochemistry, which does not consider structural changes.

A study of the dependence of adsorbabilities on the stereochemical configuration will probably acquire an increasing importance for the theoretical chemist. For the chromatographer, the problem of the dependence of the adsorption affinity on the structure finds its parallel in the problem of the dependence of the adsorption affinity on the spatial configuration.

The pertinent experimental data in this field may conveniently be divided into two parts. First, we shall consider those asymmetric compounds in which no double bond is responsible for the existence of stereoisomers; and, second, those in which *cis-trans*-isomerism is caused by the presence of double bonds.

**Optical Antipodes and Diastereoisomers.** The interesting question, "How far can racemates be resolved on the Twsett Column?" naturally arises in any consideration of compounds of the first type.

The idea that an optically active adsorbent may be used for this purpose can be traced back to an early paper of Willstätter (1904) who, two years before Tswett's first article on chromatography appeared, suggested that important information about the nature of the dyeing process might be obtained by investigating whether or not asymmetrical wool or silk fibers can be selectively dyed by one of the antipodes which are present in a racemic dye. Since no such dye was available at that time, Willstätter conducted model experiments with racemic alkaloids which can be easily adsorbed on protein fibers. The results were negative. His idea was later taken up by Porter and Ihrig (1923), who made use of some dyes which were prepared by coupling a diazo solution with racemic aminomandelic acid. Under favorable conditions, the antipodes were retained differently, and the remaining solution was optically active. However, these observations could not be confirmed by some other authors.

Recently, Martin and Kuhn (1941) passed an endless wool strip through a solution of racemic mandelic acid and observed (when a suitable temperature gradient was applied) that, as adsorption progressed, the solution acquired rotatory power.

Henderson and Rule (1936, 1939) must be credited with a chromatographic resolution of the racemate of *p*-phenylene-*bis*-iminocamphor, even if the operations were lengthy and required much adsorbent. For example, when 30 mg. of the compound was developed with petroleum ether on 6 kg. of lactose, the specific rotations $+ 485°$ and $— 728°$ were observed instead of $\pm 1500°$.

In inorganic chemistry, a partial resolution of racemic triethylenediamine chromichloride, $(\mathrm{Cr\ en_3})\ \mathrm{Cl_3}+3\ \frac{1}{2}\ \mathrm{H_2O}$, on columns made of *d*- *or* *l*-quartz was achieved by Karagunis and Coumoulos (1938), who carried out a fractional elution with 85 per cent alcohol.

It follows, from some recent reports, that the well-known method of the resolution of a racemic acid by coupling it with an asymmetric alkaloid can be made more effective by a combination with chromatographic procedures. Hass, de Vries, and Jaffé (1943) observed that a partial resolution of brucine-*d,l*-mandelate takes place when this salt is developed with benzene on glucose. Furthermore, according to Fischgold and Ammon (1941), if *d,l*-mandelic acid and an optically active alkaloid are retained simultaneously on charcoal, the unadsorbed portion of the acid shows rotatory power.

That, in principle, a separation of diastereoisomers can be carried out on optically inactive adsorbents, may be illustrated by the following examples.

Jamison and Turner (1942) developed *l*-menthyl-*d,l*-mandelate with petroleum ether on alumina. The 15 cm. top section yielded, upon saponification, a mandelic acid with $[\alpha]_D = —18.2°$, but the corresponding figure for a fourth such section was $+64°$. Stoll and Hofmann (1938) succeeded in resolving an isomorphous mixture of *d*-isolysergic acid-*d*-isopropanolamide and *l*-isolysergic acid-*d*-isopropanolamide which could not be separated otherwise. When the mixture was developed with

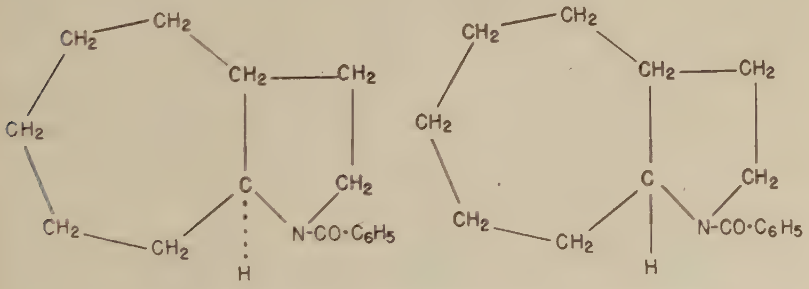

FIGURE 1. Diastereoisomeric N-benzol-cycloheptano-2,3-pyrrolidines.

acetone on alumina, the first few fractions from the liquid chromatogram yielded a crystalline *d*-derivative, $[\alpha]_D = +416°$; further portions showed first decreasing dextrorotation, then increasing levorotation, and the final fractions yielded practically pure *l*-compound, $[\alpha]_D = -342°$.

A clear separation of the two diastereoisomeric N-benzoyl-cycloheptano-2,3-pyrrolidines (FIGURE 1) was carried out by Prelog and Geyer (1945) by fractional washing on alumina with benzene and then with ether. First the α-isomer, m.p. 109°, then a mixture, and, finally, the pure β-isomer, m.p. 86-7°, passed into the filtrate.

Another recent contribution was made by Prelog and Wieland (1944). Previous to their work, it does not seem to have been possible to resolve a compound which contained an asymmetric trivalent nitrogen atom into its optically active components. These authors selected as starting material "Tröger's base" (FIGURE 2) and made use of petroleum ether on lactose in a liquid chromatogram procedure. The efficiency of this resolution is indicated by the fact that only 450 parts of sugar were necessary for 1 part of the compound, of which 5-6 per cent was resolved in a single run. After systematic recrystallizations of the highly rotating fractions, the values, $[\alpha]_D = +287°$ and $-278°$ were obtained.

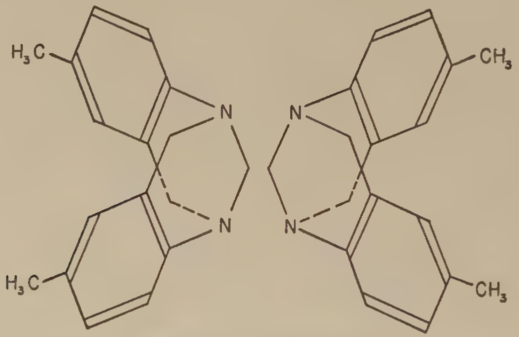

FIGURE 2. Tröger's base.

Prelog and Wieland's contribution is remarkable in two ways: first, by application of chromatography, the important stereochemical fact has been secured that, in structures like Tröger's base, an asymmetric trivalent nitrogen atom must occupy a stable position outside the plane of its three substituents; and, second, the possibility of a sequence inversion has been extended into the field of stereochemistry, since it was shown that, while a lactose column adsorbs the *l*-form of Tröger's base more strongly than it does the *d*-form, columns of *d*-tartaric acid or *d*-potassium hydrotartate prefer the *d*-isomer.

***Cis-trans* Isomerism around Nitrogen-Nitrogen or Carbon-Nitrogen Double Bonds.** It is well known that illumination of azobenzene solutions produces a lower melting isomer which is termed the *cis* form. It was shown by Cook (1938; Cook and Jones, 1939), and somewhat later by Zechmeister, Frehden, and Fischer-Jörgensen (1938) that the mixture of the two isomeric azobenzenes can be resolved on alumina by developing with benzene. The *cis* form is very well separated and occupies top position. The same sequence is exhibited by a number of azo dyes investigated by Cook. However, for *bis*-azo structures, like $C_6H_5.N{=}N.C_6H_4.N{=}N.C_6H_5$, he found the sequence from top to bottom to be *cis-trans, cis-cis,* and *trans-trans.*

The reversibility of the sequence of *cis*- and *trans*-azobenzene is implied by a study of Freundlich and Heller (1939), who showed that *cis*-azobenzene, which is the more hydrophilic isomer, is preferentially adsorbed by the more hydrophilic adsorbent, alumina. In contrast, the more hydrophobic *trans* form is the preferred one on the surface of a more hydrophobic adsorbent such as charcoal.

Examples of carbon-nitrogen double bonds which form *cis-trans* isomeric pairs are benzoin oxime, $C_6H_5.CHOH.C({=}NOH).C_6H_5$, and anisoin oxime, $C_6H_5.CHOH.C({=}NOH).C_6H_4.OCH_3$. Their *trans* forms, when developed on filtrol with benzene + 2% alcohol, are much more strongly adsorbed than the corresponding *cis* isomer. The stereoisomers can be located easily by brushing the column with ammoniacal copper sulfate. It should be mentioned that, in this instance, the *cis* configuration is not entirely stable under the conditions of the experiment and isomerizes on the column to give a few per cent of *trans* oxime (Zechmeister, McNeely, and Sólyom 1942).

***Cis-trans* Isomerism around Carbon-Carbon Double Bonds.** *Diphenylpolyenes.* In the field of the $\alpha,\omega$-diphenylpolyenes, $C_6H_5.(CH{=}CH)_n.C_6H_5$, there is some information available concerning the dependence of the adsorption affinities on the spatial configuration, especially for the members of this homologous series with $n = 1$, 2, or 4. The usual synthetic methods produce *trans* forms; these, however, under the influence of illumination or other factors suffer a *trans* → *cis* rearrangement. Subsequent chromatographic separation shows that, at least for the three compounds mentioned, the all-*trans* form possesses a greater adsorption affinity than the corresponding *cis* isomer(s).

The location of the two stereoisomeric stilbenes ($n = 1$), and some of their derivatives, can be easily carried out after a development with petroleum ether, on alumina by means of permanganate (Zechmeister and McNeely, 1942).

Diphenylbutadiene ($n = 2$) was recently investigated (Sandoval and Zechmeister, 1947). In this case, also, upon illumination, two important characteristics are altered, *viz.,* the adsorbability decreases and the extinction values are reduced. At the same time, the initial fine structure shown by the spectral curve of the all-*trans* form disappears. Both the all-*trans* and a *cis* isomer can be located on the column by means of ultraviolet light, namely, by the intense fluorescence of the former and by the quenching of the weak fluorescence of alumina at the position of the latter.

In the case of diphenyloctatetraene ($n = 4$), a similar parallelism between adsorption and extinction changes exists (Zechmeister and LeRosen, 1942). The formation of most of the nine possible stereoisomeric forms of this compound is prevented by steric hindrance. The three fluorescing zones which can be seen on the calcium hydroxide column separate well from each other when developed with benzene-petroleum ether. The all-*trans* form occupies top position and is followed probably by *trans-cis-trans-trans-* and then by *trans-cis-cis-trans-*diphenyloctatetraene.

An interesting phenomenon was observed during chromatographic experiments with this polyene. When a *cis* zone was inspected for too long a time (say, for a whole minute) with the ultraviolet lamp while development was going on, it divided into two zones, one of which was all-*trans*-diphenyloctatetraene, newly formed on the column by photoisomerization. Thus, two all-*trans* zones were present on the same column, the second one at the place of its formation. This non-intended rearrangement took place on the cylindrical surface of the adsorbent, while the inside of the column showed only the original *cis* zone but none of a locally formed all-*trans* zone. In such cases, it is practical, before the solution is introduced, to cover the chromatographic tube with black paper except for a narrow observation slit.

*Carotenoids.* We are now going to discuss some of the services which have been rendered by chromatography in studies of the stereochemistry of the naturally occurring polyenes, the carotenoids. In this widely distributed class of pigments, the molecule is characterized by an unusual number of conjugated double bonds, for example, ten in $\alpha$-carotene and eleven in $\beta$-carotene, $\gamma$-carotene, and lycopene ($C_{40}H_{56}$). For a time, it was believed by some authors that, within the framework of such a long conjugated system, each double bond loses so much of its individual character that no clearly defined *cis* and *trans* form can be expected.

As is well known, it was the chromatographic method which helped to disprove such concepts. The fundamental observation was made by Gillam and El Ridi (1936), who reported that carotene, when repeatedly chromatographed, produces a second zone, containing an isomer, below the unaltered main section on the Tswett column. The reversibility of the process was clearly recognized (Gillam and El Ridi, 1936; Gillam,

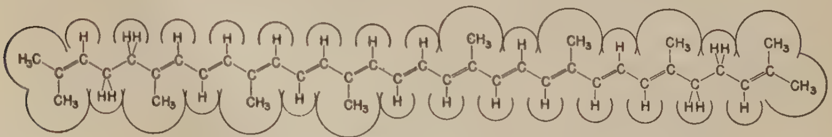

FIGURE 3. Model of all-*trans*-lycopene.

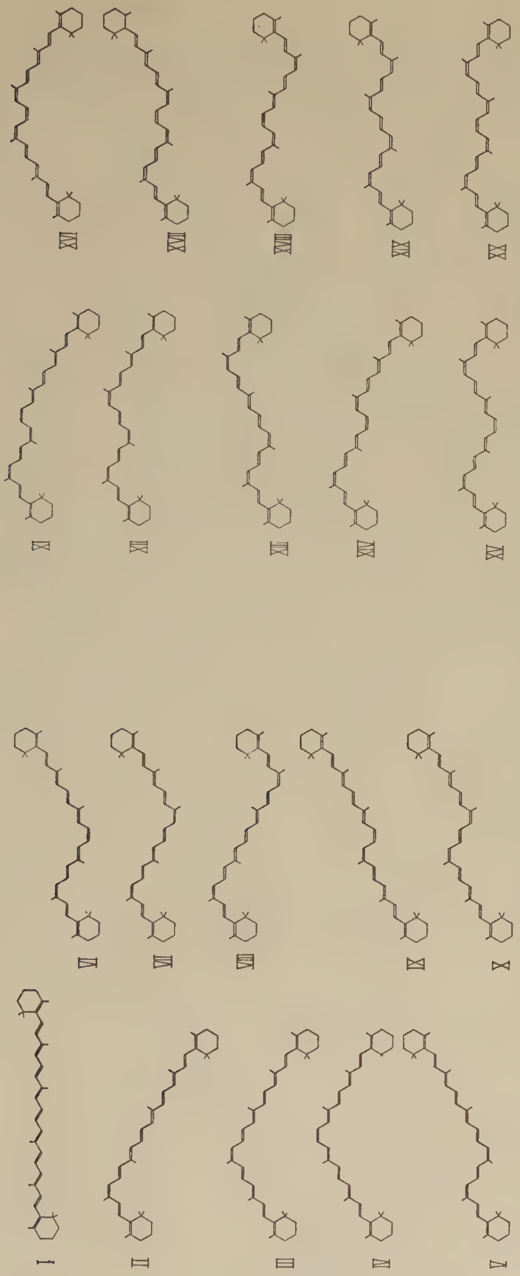

Figure 4. Skeleton models of the twenty members of the stereoisomeric β-carotene set.

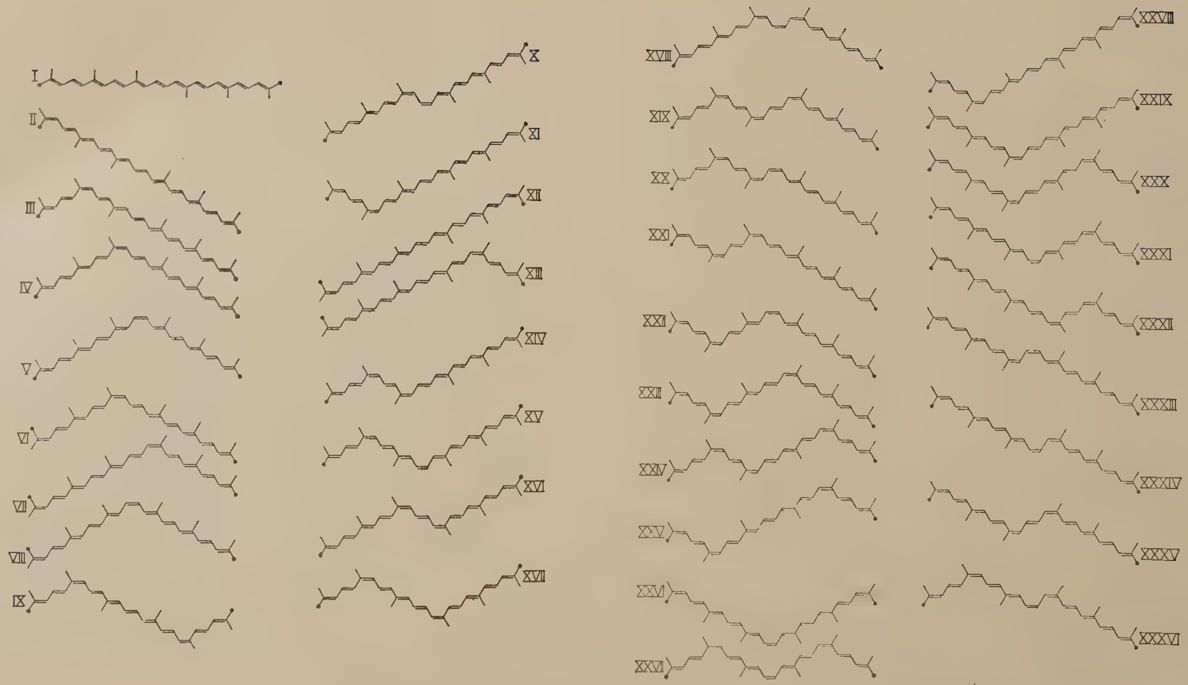

FIGURE 5. Skeleton models of the seventy-two sterically unhindered *cis-trans*-isomeric forms of lycopene (constructed by J. H. Pinckard): one all-*trans*-lycopene (I); four mono*cis*-lycopenes (II-V); twelve di*cis*-lycopenes (VI-XVII); nineteen tri*cis*-lycopenes (XVIII-XXXVI); nineteen tetra*cis*-lycopenes (XXXVII-LV); twelve penta*cis*-lycopenes (LVI-LXVII); four hexa*cis*-lycopenes (LXVIII-LXXI); and one hepta*cis*-lycopene (LXXII). The single side lines represent methyl groups; the black circles stand for the carbon skeleton of the end groups in the lycopene molecule.

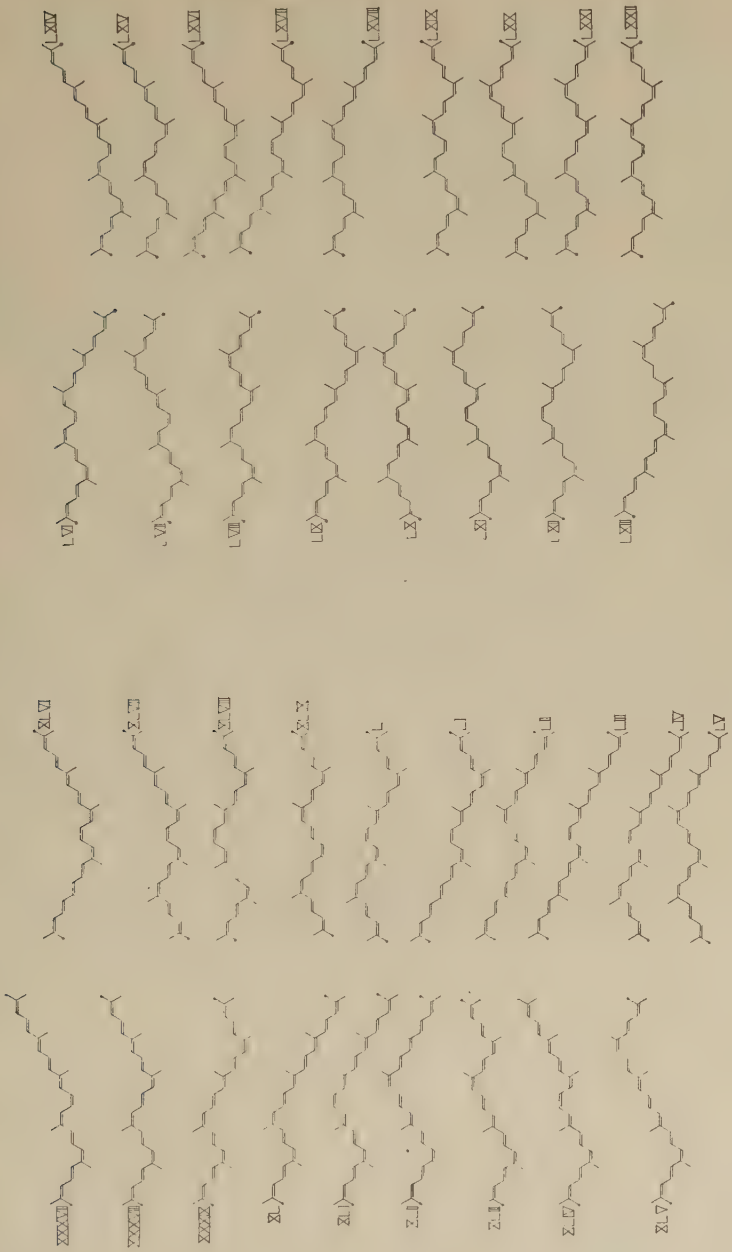

FIGURE 5 (*continued*).

El Ridi, and Kon, 1937). The isomerization of some carotenoids was also described, somewhat later, by the author and Cholnoky (1937), as well as by Strain (1938, 1941). It was shown by the author and Tuzson (1938) that this isomerization is independent of the adsorption process (confirmed by Carter and Gillam, 1939); and, furthermore, that the classic catalyst for *cis-trans* rearrangements, iodine, can be used also in the field of the $C_{40}$-carotenoids (1939; *cf.* Zscheile, Harper, and Nash, 1944. For a more detailed evaluation of the literature on isomerization methods, see a review written by the author, 1944; *cf.* Strain, 1938, 1939).

By far the major fraction of the naturally occurring carotenoid molecules is in the all-*trans* configuration (FIGURE 3), and even those equilibria or quasi-equilibria which can be produced by thermal, photochemical, or catalytic processes mostly contain the all-*trans* form as a main constituent and partially *cis* forms in lesser amount. The totality of all possible stereoisomers of a polyene is termed a "stereoisomeric set", and each isomer is a "member" of the set.

Calculation shows that, even for a relatively simple symmetrical compound such as $\beta$-carotene, the stereoisomeric set should include 272 members. However, according to Pauling (1939), four of the nine aliphatic double bonds cannot assume *cis* configuration, because of steric hindrance which is caused by the presence of methyl side chains. The expected number of stereoisomers is thus reduced to twenty (FIGURE 4). Even so, there is, evidently, no other method but chromatography which is applicable to the separation of stereoisomeric $\beta$-carotenes, of which about a dozen have been observed so far (Polgár and Zechmeister, 1942). The same statement is valid for the stereoisomeric forms of lycopene (FIGURE 5).

It is not appropriate, at this time, to discuss the methods which may be used to assign a tentative configuration to such isomers. A few remarks may therefore suffice (for a more detailed survey, *cf.* Zechmeister, 1944). Since each *trans* → *cis* rotation involves a shift in position of the spectral maxima to shorter wavelengths relative to the all-*trans* isomer,

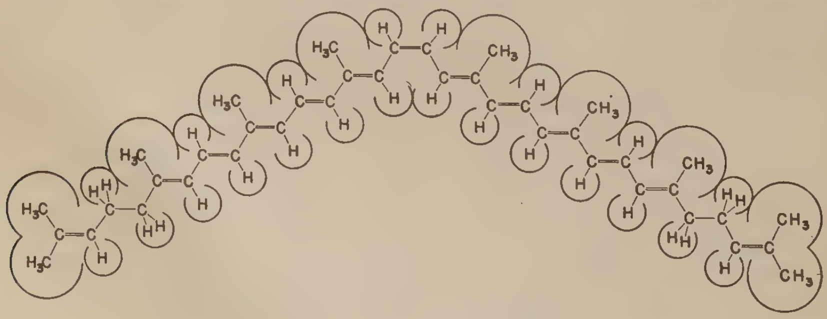

FIGURE 6. Model of central mono*cis*-lycopene.

the number of *cis* double bonds may be roughly determined. On the other hand, the so-called *cis*-peak effect, *i.e.,* the appearance of a new spectral maximum somewhere between 320 and 380 m$\mu$ (Zechmeister and Polgár, 1943) yields some information about the general shape of the molecule, that is, about the position of the *cis* double bonds. Those isomers possessing the highest *cis*-peaks are supposed to be V-shaped (FIGURE 6): in other words, a *cis* double bond is probably located at or near the center of the chromophore. In contrast, in isomers possessing low *cis*-peaks, the bending of the molecule has taken place at a peripheral section of the conjugated system (Zechmeister, LeRosen, Schroeder, Polgár, and Pauling, 1943).

Experiment has shown, in the case of carotenes, that bending of the molecule around a double bond may either increase or decrease the adsorption affinity. No parallelism is evident, in this case, between the direction of chromatographic and spectroscopic changes. That particular type of isomer, like neo-$\beta$-carotene U, which is rather stable and in which a centrally located *cis* double bond is assumed to be absent, possesses a higher adsorption affinity than the all-*trans* form, while the type of neo-B which shows a high *cis*-peak and greater liability is located below all-*trans*-$\beta$-carotene on the Tswett column.

The differences in adsorption affinity just mentioned are very considerable. In fact, they are of the same order of magnitude as effects which are caused by some structural alterations, *e.g.,* the addition or removal of a conjugated double bond. Thus, if a mixture of $\alpha$- and $\beta$-carotene is catalyzed with iodine, in light, subsequent development with petroleum ether on calcium hydroxide demonstrates considerable overlapping of the two stereoisomeric sets, as shown by the following sequence (Zechmeister and Polgár, 1944):

(*top*) Neo-$\beta$-carotene U
Neo-$\beta$-carotene V
Neo-$\alpha$-carotene U
All-*trans*-$\beta$-carotene
Neo-$\alpha$-carotene V
Neo-$\beta$-carotene B
Neo-$\beta$-carotene E
Neo-$\alpha$-carotene W
Neo-$\beta$-carotene F
All-*trans*-$\alpha$-carotene
Neo-$\alpha$-carotene B
(*bottom*) Neo-$\alpha$-carotenes C, D, etc.

In connection with these observations, it was desirable to investigate how far the adsorption affinities are influenced by a great number of *cis* double bonds, and whether or not the rotation of, say, four to seven double bonds into *cis* position involves parallel or opposite changes in adsorbabilities and position of the spectral bands. Unfortunately, no method is available, at the present time, which permits the preparation *in vitro* of poly*cis* carotenoids. Evidently, the probability of the formation of such stereoisomers is very low under the experimental conditions applied. However, nature has provided us with the desired material in the form of several crystallized poly*cis* compounds, *e.g.*, prolycopene (FIGURE 7) and pro-γ-carotene, $C_{40}H_{56}$, which occur in several fruits (Zechmeister, LeRosen, Went, and Pauling, 1941; Zechmeister and Schroeder, 1942; LeRosen and Zechmeister, 1942; in petals: Schroeder, 1942). Recently, in collaboration with Pinckard (1947), six more poly*cis* lycopenes were observed which doubtlessly contain four to seven *cis* double bonds.

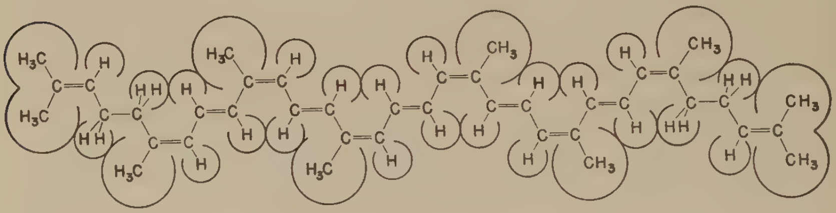

FIGURE 7. Model of prolycopene (a hexa*cis*-lycopene).

All of these compounds show a flat spectral curve in the *cis*-peak region, and a very much decreased adsorption affinity compared with that of the corresponding all-*trans* pigment or any stereoisomer containing a few *cis* double bonds as obtained *in vitro*. Evidently, when all or most of the sterically available double bonds are in *cis* configuration, the increasing or decreasing effect of a single *trans* → *cis* shift is overruled by the total effect of the poly*cis* configuration.

Our knowledge is less complete in the field of the dihydroxycarotenes, *e.g.*, lutein or zeaxanthin, $HO.C_{40}H_{54}.OH$, where no poly*cis* forms are available. As found by Strain (1938) and, independently, by our group (Zechmeister and Cholnoky, 1937; Zechmeister and Tuzson, 1939), all stereoisomers of the dihydroxy carotenes are adsorbed considerably above the all-*trans* form. This is also true for the important algal pigment fucoxanthin, $C_{40}H_{56}O_6$, as pointed out by Strain and Manning (1942).

Whether or not the increased adsorption affinity of *cis* xanthophylls is caused by the diminished distance between the two hydroxyl groups, cannot be decided at the present time. In favor of such an interpretation is the fact that the monohydroxy compound, cryptoxanthin, $HO.C_{40}H_{55}$, behaves like the hydrocarbons and yields stereoisomers with both increased and diminished adsorbability (Zechmeister and Lemmon, 1944).

The contrast in the behavior of a polyene hydrocarbon and the corre-

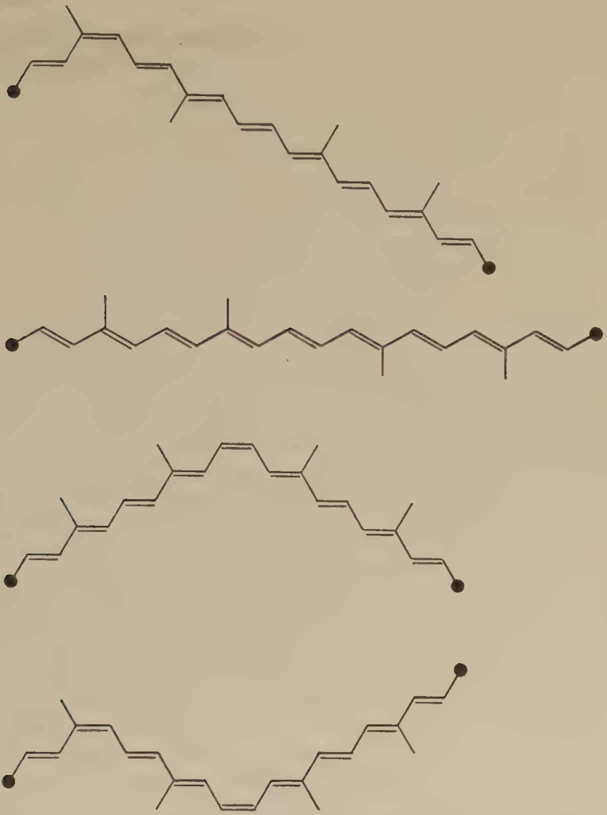

FIGURE 8. Skeleton models of four crystallizable stereoisomeric methylbixins, in the order of decreasing adsorption affinities (the dots designate —$COOCH_3$ groups): natural methylbixin (top); all-*trans*-methylbixin; neomethylbixin A; and neomethylbixin C (bottom).

sponding dihydroxy derivative can be illustrated, for example, by the following chromatographic sequences:

| | *α-Carotene Set:* | *Dihydroxy-α-Carotene Set:* |
|---|---|---|
| (*top*) | peripherally bent isomers | V-shaped isomer |
| | all-*trans* form | peripherally bent isomer |
| (*bottom*) | V-shaped isomers | all-*trans* form |

A lower molecular weight polyene, the dicarboxylic methylester, methylbixin, $CH_3OOC.C_{22}H_{26}.COOCH_3$, shows in principle the behavior of the carotenes, as illustrated by FIGURE 8 (Zechmeister and Escue, 1944).

We may summarize the available information in the field of *cis-trans* isomeric compounds containing sterically effective carbon-carbon double bonds as follows.

The adsorption affinities are highly dependent on the spatial configuration, often more so than on some structural features. If only one or two double bonds are present, classic procedures like crystallization may be used for the isolation of the stereoisomers. However, with a progressing lengthening of the unsaturated system and, thus, an increase of the number of the members of the set, chromatography becomes the only practical method for the resolution of a complicated stereoisomeric mixture. It seems even that the future development of certain chapters of stereochemistry will depend on the expert use of adsorption procedures.

A general theory of the dependence of the adsorption affinities on the spatial configuration is not available, and nothing is known concerning the anchoring points and the orientation of the individual stereoisomeric molecules on the active surface. On the other hand, the influence of *trans* → *cis* rearrangements on the chromatographic behavior is now better defined than previously, especially in the field of some natural polyene pigments.

Since the chromophoric group is responsible also for the spectrum, combined spectroscopic and chromatographic observations may be of considerable use in the future. Under the influence of spatial rearrangements, adsorbability and light extinction may undergo changes in a parallel or in an opposite direction. A closer study of such phenomena may offer an approach both for the interpretation of spectra and of adsorption processes. These problems are, however, of much deeper significance than those which could be discussed in the present short review of the relationship between stereochemistry and chromatography.

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# CHROMATOGRAPHY IN THE STREPTOMYCIN PROBLEM

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Streptomycin has become increasingly important since its discovery was first reported, in 1944, by Schatz, Bugie, and Waksman.[1] The isolation of pure streptomycin and the application of streptomycin to clinical practice in less than three years after its discovery represent a significant achievement. To a considerable extent, this accomplishment is due to the development of chromatographic methods for purification of the active substance. The present paper is concerned with this phase of the streptomycin problem.

The object of the reported work on the chromatographic purification of streptomycin was essentially the preparation of pure material for degradative and other studies, rather than the careful investigation of chromatographic behavior. It will be understandable, therefore, that there remains much to be desired concerning knowledge of the chromatographic behavior of streptomycin.

The problem was the separation of a complex active substance which was soluble in water and in methanol, but nearly insoluble in the common organic solvents, from large amounts of inactive impurities possessing very similar solubility properties. Chromatography appeared to be the only practicable approach, since neither purification by extraction from water into organic solvents and back into water, nor direct crystallization of the active substance was possible. The solubility of streptomycin concentrates was such that the solvent for chromatography was necessarily strongly polar (water, methanol, or mixtures largely composed of these with miscible solvents). Examination of adsorption behavior led to the general choice of alumina as the adsorbent for columns. Darco G-60 carbon was also found applicable.

The colorless nature and similarity in properties of streptomycin and many of the impurities present in the concentrates made it difficult to follow the chromatographic purification of streptomycin on the columns by visual observation. Although colored salts of streptomycin have been made,[2, 3] their chromatography does not appear to have been investigated to any great extent. The "liquid chromatogram", discussed by Zechmeister and Cholnoky,[4] has been employed, therefore, for the chromatographic work thus far described in detail.

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The liquid chromatogram, in principle, consists of a series of filtrates successively collected from a chromatographic column. In this type of procedure, part or all of the adsorbate is forced through the column into the filtrate. Examination of the arbitrary series of filtrate fractions taken gives the required information on fractionation.

Investigation of liquid chromatograms of streptomycin has been based on concentration of solids and concentration of activity in the filtrate fractions. Other variables, such as optical rotation and nitrogen content, could also be followed. Plotting these results against the filtrate volumes gives a graphic picture of the chromatogram.

A brief discussion of the source and chemical nature of streptomycin is given here, in order to furnish a basis for an understanding of the type of compound being purified by chromatography. Streptomycin is produced by the mold, *Streptomyces griseus,* grown under certain specified conditions.[1] The antibiotic is usually recovered[2, 3, 5, 6, 7] from filtered culture broths by adsorption on activated charcoal. The charcoal is eluted with acid alcohol and the antibiotic is obtained as a crude, amorphous, hygroscopic powder. This concentrate may be purified by chromatography to yield nearly pure streptomycin salts.

Streptomycin preparations from chromatographic purification have been converted to pure crystalline salts, including the reineckate,[2, 5] helianthate,[3] sulfate,[3] *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonate,[3] and the calcium chloride double salt.[8] From investigations carried out on the pure salts, and on degradation products obtained from them, much of the chemical nature of streptomycin has become known. Streptomycin is a levorotatory base, $C_{21}H_{39}N_7O_{12}$, with a molecular weight of 582. The trihydrochloride shows $[\alpha]_D$ —86.7°. Streptomycin appears to be a glycoside composed of a 1,3-diguanido-2,4,5,6-tetrahydroxycyclohexane, termed streptidine,[9] and a disaccharide-like substance designated streptobiosamine.[10] Streptobiosamine, $C_{13}H_{23}NO_9$, is composed of N-methyl-l-glucosamine and a six-carbon sugar-like fragment, designated streptose[10] or streptonose.[11] The reducing group of the N-methyl-l-glucosamine fragment is bound in glycosidic linkage to streptose in streptobiosamine. There appears to be an aldehydic group in the streptose portion of intact streptomycin,[11] and also a C-methyl group.[12] Reaction of streptomycin

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[5] **Carter, H. E., R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell, & W. A. Strong.** J. Biol. Chem. **160**: 337. 1945.

[6] **Le Page, G. A., & E. Campbell.** J. Biol. Chem. **162**: 163. 1946.

[7] **Vander Brook, M. J., A. N. Wick, W. H. De Vries, R. Harris, & G. F. Cartland.** J. Biol. Chem. **165**: 463. 1946.

[8] **Peck, R. L., N. G. Brink, F. A. Kuehl, Jr., E. H. Flynn, A. Walti, & K. Folkers.** J. Am. Chem. Soc. **67**: 1866. 1945.

[9] For leading references to the structure of streptidine, see: **Peck, R. L., C. E. Hoffhine, Jr., R. P. Graber, F. W. Holly, R. Mozingo, & K. Folkers.** J. Am. Chem. Soc. **68**: 776. 1946.

[10] For leading references to the structure of streptobiosamine, see: **Kuehl, F. A., Jr., E. H. Flynn, N. G. Brink, & K. Folkers.** J. Am. Chem. Soc. **68**: 2096. 1945; and **Brink, N. G., F. A. Kuehl, Jr., E. H. Flynn, & K. Folkers.** J. Am. Chem. Soc. **68**: 2405. 1946.

[11] **Fried, J., & O. Wintersteiner.** Abstracts of the Chicago Meeting, A.C.S., Sept., 1946: 15 B.

[12] **Hooper, I. R., L. H. Klem, W. J. Polglase, & M. L. Wolfram.** J. Am. Chem. Soc. **68**: 2120. 1946.

with hydroxylamine and with semicarbazide has been shown to occur.[13] Reduction of streptomycin gives rise to dihydrostreptomycin,[14] a biologically active substance, while oxidation yields biologically inactive streptomycinic acid.[11] These reactions apparently involve only the aldehydic group. A diagrammatic representation of the structure of streptomycin is shown in FIGURE 1.

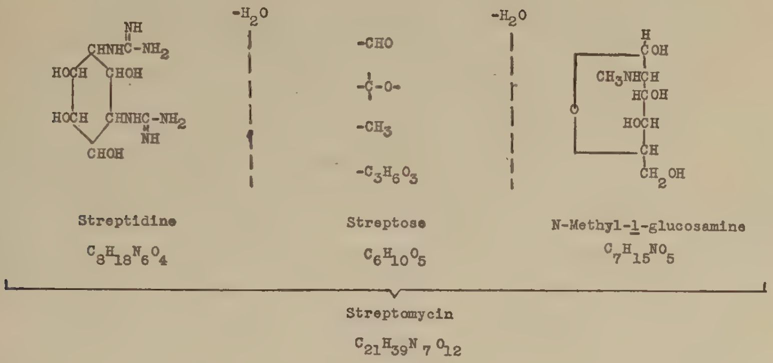

FIGURE 1.

Streptomycin trihydrochloride is quite soluble in both water and methanol, and slightly soluble in ethanol. The sulfate is quite soluble in water but only slightly soluble in methanol. Aqueous solutions of pure streptomycin trihydrochloride are slightly acidic. The activity of streptomycin trihydrochloride is about 800 units per mg., although the values obtained depend to a certain extent on the methods of assay.

Fried and Wintersteiner[2] first reported the preparation of crystalline streptomycin reineckate from concentrates of streptomycin purified by steps including chromatography. From their brief description, it appears that streptomycin picrate was employed for the chromatographic purification. The reineckate assayed about 400 units per mg., and streptomycin sulfate prepared from the pure reineckate assayed about 850 units per mg. Shortly afterwards, there appeared a paper by Kuehl, Peck, Walti, and Folkers[3] which described crystalline salts of streptomycin, including the helianthate, *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonate, and sulfate, which were also prepared from streptomycin concentrates obtained by chromatographic procedures.

Carter and his associates[5] first described in detail a chromatographic purification procedure for the preparation of highly active concentrates of streptomycin. This method employed alumina as adsorbent and 80 per cent methanol as developing solvent. The streptomycin concentrate used in the chromatograms had an activity of 150-300 units per mg. It was found that alkaline alumina removed streptomycin from neutral aqueous solution, but elution with aqueous acid was slow and incomplete. Acid-washing alumina did not remove streptomycin from neutral aqueous solution, but did so from aqueous methanol. The chromatographic method used was developed on the basis of this information. It is to be noted that solutions of the concentrates of streptomycin hydrochloride were partially neutralized before introduction to the columns.

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[13] Brink, N. G., F. A. Kuehl, Jr., & K. Folkers. Science 102: 506. 1945.
[14] Peck, R. L., C. E. Hoffhine, Jr., & K. Folkers. J. Am. Chem. Soc. 68: 1390. 1946.

Crude streptomycin hydrochloride in 70-80 per cent methanol at a pH of about 6.3 was percolated over a sulfuric acid-washed alumina column (pH 5-6). An inactive filtrate fraction giving a positive Sakaguchi test for guanido groups was followed by a fraction giving a negative Sakaguchi test. The subsequent fractions gave positive and strong such tests. The rapid rise of this test to a peak paralleled the increase in activity of the fractions. A small amount of activity remained on the columns. This could be removed by lowering the methanol content of the developing solvent. The material so eluted contained sulfate ion but no chloride ion. The evidence indicated that chloride ion was replaced by sulfate ion on the column, an exchange which rendered the product (streptomycin sulfate) less soluble in the developing solvent (70-80 per cent methanol), and thereby caused it to require the use of water for elution. Streptomycin sulfate is known to be much less soluble than the hydrochloride in methanol. The most active fractions from the column were concentrated to remove methanol, and lyophilized to give amorphous powders ranging in activity from 600-900 units* per mg. Less active fractions could be purified by repetition of the chromatographic procedures.

The alumina used (Merck or Harshaw) had been acidified to pH 6 with 50 per cent sulfuric acid and back washed with distilled water until the washings were sulfate-free. The pH of the final wash was 6.0. The alumina was put into the column as a slurry in water, then thoroughly washed with 80 per cent methanol. Columns, 3.2 cm. in diameter, containing 480 cc. of packed alumina were used for 8-10 gram lots of streptomycin. The weight ratio of adsorbent to solute is estimated to be about 25-50:1.

The crude streptomycin hydrochloride (8-10 g.) was dissolved in 90 cc. of 80 per cent methanol and brought to pH 6.3 with 2.0 *N* lithium hydroxide in 80 per cent methanol. The slight precipitate was removed by filtration, and the filtrate was carefully poured onto the prepared column. The chromatogram was developed with 80 per cent methanol. The solution leaving the column was collected in 100-cc. fractions. Sakaguchi and chloride tests were made to check the location of the fractions. When the Sakaguchi test began to decrease, the 80 per cent methanol was replaced with water as developing solvent. This accounts for the observation of secondary peaks of activity (see FIGURE 2). Various fractions were combined for preparation of solid products. TABLE 1 gives data on these fractions. Based on the activity of pure streptomycin hydrochloride,[2, 3] the best fractions from such columns were probably at least 90 per cent pure streptomycin hydrochloride. TABLE 2 shows the

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* In such an instance, it is believed that comparative assays with pure streptomycin trihydrochloride would show the latter to be more active than 900 units per mg.

TABLE 1*

SOLID FRACTIONS FROM ALUMINA COLUMN

| Fraction No. | Weight of solid g. | Activity of solid units per mg. | Nitrogen content per cent |
|---|---|---|---|
| 7-11 | 0.45 | 600 | 11.40 |
| 12-17 | 1.00 | 900 | 13.47 |
| 18-23 | 0.65 | 620 | 13.83 |
| 24-26 | 0.43 | 520 | |

* From Carter, H. E., R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell & W. A. Strong. J. Biol. Chem. 160: 337. 1945.

TABLE 2*

FRACTIONATION OF CRUDE STREPTOMYCIN ON ALUMINA COLUMN

| Fraction No. | Volume cc. | Sakaguchi | Chloride | Activity units per cc. | Total units |
|---|---|---|---|---|---|
| 2 | 800 | — | + | 0 | |
| 3 | 100 | — | + | 0 | |
| 4 | 100 | + | + | 0 | |
| 5 | 100 | + | + | 0 | |
| 6 | 100 | + | + | 0 | |
| 7 | 100 | — | — | 16 | 1,600 |
| 8 | 100 | + | + | 170 | 17,000 |
| 9 | 100 | + | + | 400 | 40,000 |
| 10 | 100 | + | + | 570 | 57,000 |
| 11 | 100 | ++ | + | 800 | 80,000 |
| 12 | 100 | +++ | + | 1140 | 114,000 |
| 13 | 100 | +++ | + | 1140 | 114,000 |
| 14 | 100 | +++ | + | 1140 | 114,000 |
| 15 | 100 | +++ | + | 1340 | 134,000 |
| 16 | 100 | +++ | + | 1340 | 134,000 |
| 17 | 100 | +++ | + | 1140 | 114,000 |
| 18 | 100 | ++ | + | 960 | 96,000 |
| 19 | 100 | ++ | + | 920 | 92,000 |
| 20 | 100 | ++ | + | 570 | 57,000 |
| 21 | 100 | ++ | + | 720 | 72,000 |
| 22 | 100 | ++ | + | 1060 | 106,000 |
| 23 | 100 | + | + | 570 | 57,000 |
| 24 | 200 | + | + | 800 | 160,000 |
| 25 | 200 | + | — | 380 | 76,000 |
| 26 | 200 | + | — | 400 | 80,000 |
| *Total units recovered* | | | | | 1,715,000 |

* From Carter, H. E., R. K. Clark, Jr., S. R. Dickman, Y. H. Loo, P. S. Skell & W. A. Strong. J. Biol. Chem. 160: 337. 1945.

fractionation data obtained in the chromatogram described above. The streptomycin hydrochloride put onto this column weighed 9.9 g., had an activity of 221 units per mg., and contained a total of 2.19 million units.

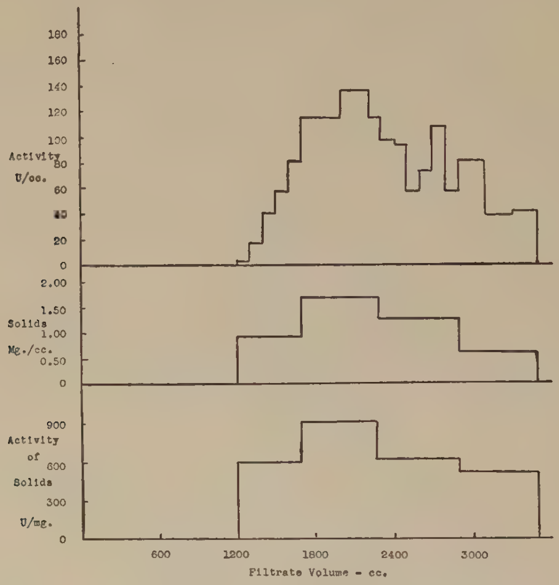

**Figure 2.** Chromatogram of streptomycin hydrochloride, 221 units/mg. Solvent: 80% methanol; adsorbent: alumina. (Data from Carter et al.[5])

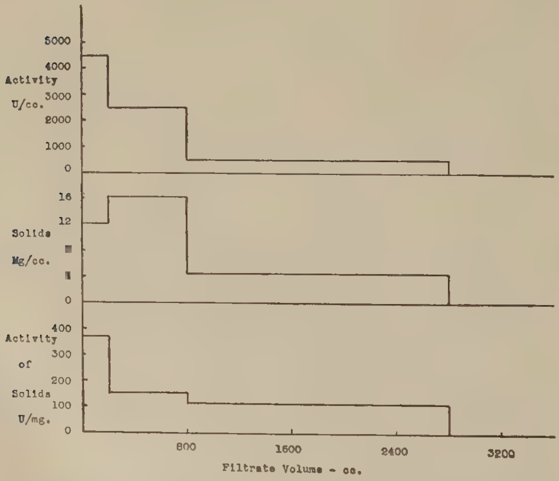

**Figure 3.** Chromatogram of streptomycin hydrochloride, 72 units/mg. Solvent: methanol; adsorbent: alumina. (Data from Kuehl et al.[15])

FIGURE 2 gives a graphic study of this chromatogram. The solid content of the filtrate is quite low, reaching a maximum of only about 1.7 mg. per cc. (fractions 12-17). The activity maximum in the filtrate was about 1340 units per cc. The second maximum of activity in FIGURE 2 is the result of change from 80 per cent methanol to water after fraction 20 had been taken. It will be observed, from the graphs, that the most active fractions were obtained at the point of maxima of both activity and solids concentration. Lack of data on solids concentration makes it hard to judge the degree of coincidence of these maxima. It is to be noted that considerable solvent was collected before much solid appeared in the filtrate. Lacking other data, this indicates a moderately slow passage of the solute through the column, relative to solvent flow. Since streptomycin is a base, the lower the pH of its solutions the more ionized it will be and, consequently, the less strongly adsorbed. The slow passage of the active material through this column, therefore, may be ascribed in part to its partial neutralization. Slow passage through the column should favor effective purification. Approximately four-fold purification was achieved in this experiment, and the best fractions were very close to purity, in so far as they may be judged by bioassay data.

Kuehl and associates[15] recently published details of their chromatographic purification procedures. They used absolute methanol solutions of streptomycin hydrochloride concentrates without preliminary neutralization, and absolute methanol as developing solvent. Darco G-60 carbon and sulfuric acid-washed alumina were employed as adsorbents. Filter paper pulp was mixed with the Darco G-60 for bulking purposes.[16] The filtrate fractions were collected and mixed with 5-10 volumes of acetone or ether to effect precipitation of the streptomycin hydrochloride. The products were collected by centrifugation, washed, and dried *in vacuo*. TABLE 3 gives some examples of the data obtained.

In FIGURES 3, 4, and 5 graphic studies of these results are shown. The maximum solids concentration in the filtrate fractions from these chromatograms is in the range of 7-16 mg. per cc., roughly 5-10 times as high as in the case of the column described by Carter *et al.*[5] The maximum activity concentration is in the range of 3000-8500 units per cc., again considerably higher than in the previously described case; also, this maximum occurs before the attainment of the maximum of solids. Owing, probably, to the lower pH used in these chromatograms, the active material passes down the column faster than in the preceding experiment.

In FIGURE 3, the activity maximum appears before the maximum of solids concentration, indicating a significant separation of streptomycin from one or more impurities. Separation of two zones is approximated. The concentrate employed in this column contained roughly only ten per

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[15] **Kuehl, F. A., Jr., R. L. Peck, C. E. Hoffhine, Jr., R. P. Graber, & K. Folkers.** J. Am. Chem. Soc. 68: 1460. 1946.

[16] **Wachtel, J. & H. G. Cassidy.** Science 95: 233. 1942.

TABLE 3

CHROMATOGRAPHIC PURIFICATION OF STREPTOMYCIN HYDROCHLORIDE

| Solute | | Adsorbent | | | Products | | |
|---|---|---|---|---|---|---|---|
| Wt. g. | Activity units/mg. | Wt. g. | Type | Eluate vol. cc. | Wt. of fraction g. | Activity units/mg. | Per cent of activity recovered |
| 45 | 72 | 800 | Alumina | 200 | 2.42 | 372 | 28 |
| | | | | 600 | 9.67 | 154 | 46 |
| | | | | 2000 | 8.67 | 113 | 30 |
| 6.0 | 225 | 160 | Alumina | 250 | 0.698 | 500 | 23 |
| | | | | 150 | 1.031 | 490 | 34 |
| | | | | 250 | 0.729 | 290 | 14 |
| | | | | 350 | 0.413 | 160 | 4 |
| 2.75 | 350 | 160 | Darco G-60 filter paper pulp 10:1 mixture | 25 | 0.202 | 780 | 16 |
| | | | | 25 | 0.355 | 600 | 21 |
| | | | | 50 | 0.820 | 400 | 33 |
| | | | | 50 | 0.367 | 220 | 8 |
| | | | | 250 | 0.208 | 210 | 4 |

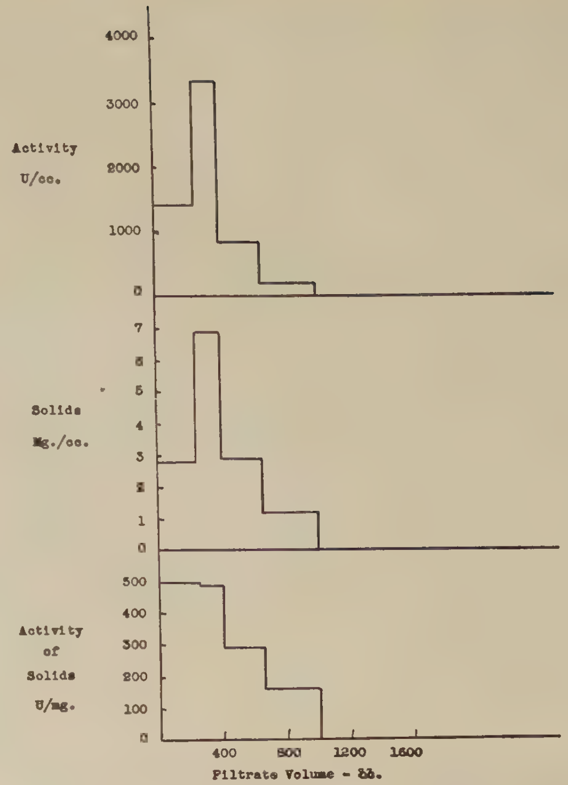

FIGURE 4. Chromatogram of streptomycin hydrochloride, 225 units/mg. Solvent: methanol; adsorbent: alumina. (Data from **Kuehl et al.**[15])

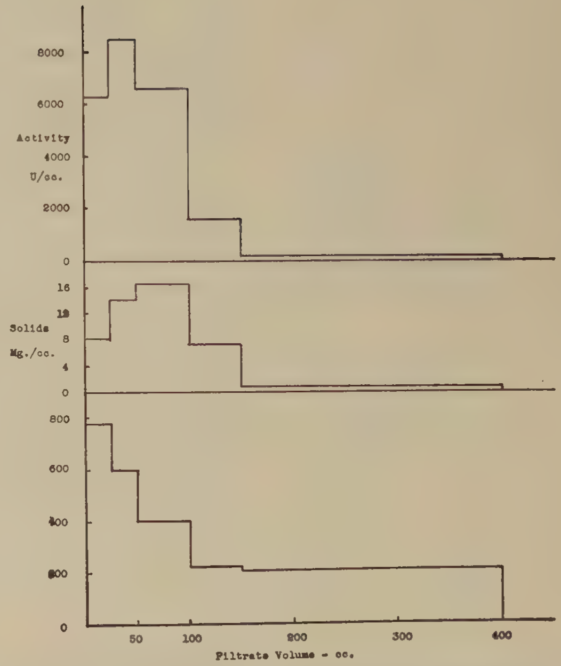

FIGURE 5. Chromatogram of streptomycin hydrochloride, 350 units/mg. Solvent: methanol; adsorbent: Darco G-60. (Data from **Kuehl et al.**[15])

cent of streptomycin, and the streptomycin appeared to be about the most rapidly moving component of the mixture. It is pointed out that, if there are salts present, *e.g.*, sodium chloride, they will move nearly as fast as the solvent, and faster than streptomycin. Their rate relative to solvent in this system[17] approaches unity; $R \cong 1$. FIGURE 4, a chromatogram of a purer concentrate on alumina, shows no definite separation of maxima. FIGURE 5, however, a chromatogram employing Darco G-60 carbon, again shows this separation. It is possible that the carbon effects greater separation of the components of streptomycin concentrates than does alumina. Its use on a large scale is, however, beset with certain practical difficulties.

Vander Brook and associates[7] have recently described a chromatographic procedure similar to that of Carter *et al.*[5] Their modification lies in the use of hydrochloric acid-washed alumina which eliminates the streptomycin sulfate zone noted[5] in the chromatogram on sulfuric acid-washed alumina. The procedure employed was the following. Five kilograms of Harshaw alumina (No. 2-350, catalyst) were adjusted to pH 4.7 with hydrochloric acid and thoroughly washed with distilled water. The acid-adjusted aluminum oxide was then suspended in 80 per cent methanol and poured, as a slurry, into a glass pipe, 4 inches in diameter, producing, after settling, a column 25 inches high.

A solution of 64 million units of streptomycin hydrochloride (160 g., 400 units per mg.) in water was brought to pH 5.8 in a final volume of 350 cc. The aqueous solution was diluted with 4 volumes of methanol before passage over the column. The weight ratio of adsorbent to solute was about 30:1. Development was carried out with 80 per cent methanol. The filtrate fractions were concentrated *in vacuo* to remove the alcohol, then dried from the frozen state. The results are shown in TABLE 4.

TABLE 4*

CHROMATOGRAPHIC SEPARATION OF 64 MILLION UNITS OF STREPTOMYCIN HYDROCHLORIDE (400 units per mg.) OVER ALUMINA COLUMN

| Fraction No. (300 cc. each) | Units $10^6$ | Solids g. | Purity units per mg. | Recovery per cent |
|---|---|---|---|---|
| 10 | 4.3 | 5.12 | 840 | 6.7 |
| 11 | 20.0 | 20.17 | 995 | 31.2 |
| 12 | 17.4 | 19.85 | 879 | 27.2 |
| 13 | 9.6 | 16.00 | 600 | 15.0 |
| 14 | 6.7 | 14.10 | 475 | 10.5 |

* From Vander Brook, M. J., A. N. Wick, W. H. De Vries, R. Harris, & G. F. Cartland. J. Biol. Chem. 165: 463. 1946.

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[17] Le Rosen, A. L. J. Am. Chem. Soc. 64: 1905. 1942.

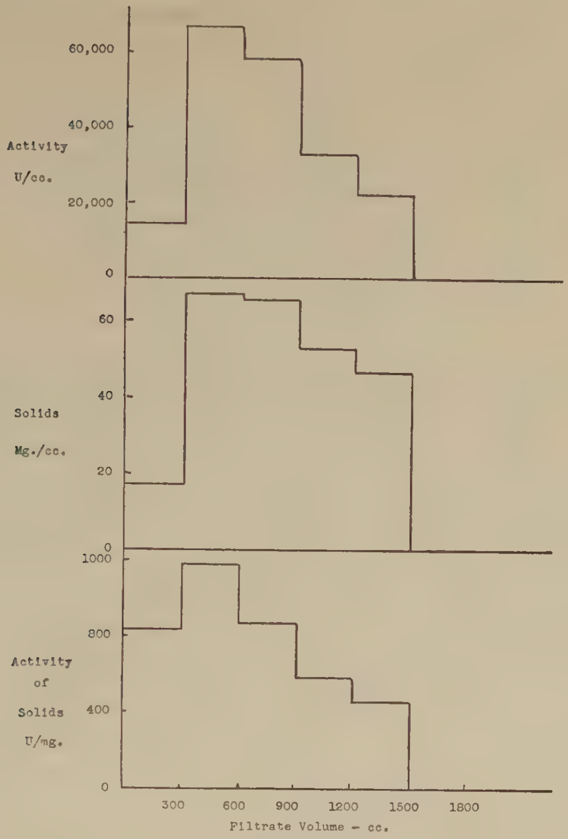

**FIGURE 6.** Chromatogram of streptomycin hydrochloride, 400 units/mg. Solvent: 80% methanol; adsorbent: alumina. (Data from Vander Brook et al.[7])

A graphic study of this column is shown in FIGURE 6. A very high concentration of material was used for this chromatogram. The solids in the filtrate attained a maximum of nearly 70 mg. per cc., and the filtrate activity reached a maximum value of about 67,000 units per cc. In this column, as in those previously described, the activity maximum appears to be ahead of the solids maximum. However, probably because of the high concentration of solute and its fairly high activity, this is not so obvious as in some of the other examples. The passage of active material was fairly slow, as judged by the fact that significant amounts of active material first appeared in fraction 10. The pH of the solution put onto this column was close to 6.0; hence, slow passage and very effective purification would be anticipated. In this chromatogram, fractions of streptomycin which were very nearly pure were obtained. This example is a good illustration of the preparative application of the chromatographic purification of streptomycin.

The chromatographic purification of streptothricin concentrates has been described by Peck *et al.*[18] The results obtained are of interest for comparison with the results of the chromatographic purification of streptomycin on alumina and Darco G-60. The procedures used were essentially identical with those described by Kuehl *et al.*[12] and were, in fact, the basis for the latter method. TABLE 5 shows the data obtained, and FIGURES 7 and 8 give graphic analyses of the data.

TABLE 5

CHROMATOGRAPHIC PURIFICATION OF STREPTOTHRICIN HYDROCHLORIDE

| Solute | | Adsorbent | | | Products | | |
|---|---|---|---|---|---|---|---|
| Wt. g. | Activity units/mg. | Wt. g. | Type | Eluate vol. cc. | Wt. of fraction g. | Activity units/mg. | Per cent of activity recovered |
| 10.0 | 49 | 160 | Alumina | 148 | 2.30 | 20 | 4 |
| | | | | 30 | 0.76 | 64 | 10 |
| | | | | 402 | 2.48 | 112* | 57 |
| | | | | 452 | 0.55 | 81* | 9 |
| 2.76 | 120 | 77 | Darco G-60 filter paper pulp (42:35) | 70 | 0.959 | 40* | 13 |
| | | | | 42 | 0.570 | 131* | 23 |
| | | | | 43 | 0.381 | 186* | 21 |
| | | | | 152 | 0.431 | 233* | 28 |

* Averaged values of composite fractions.

The maxima of solids content (14 and 25 mg. per cc., respectively, for Darco G-60 and alumina) and the maxima of activity (about 1800 and 7000 units per cc., respectively) are in about the same range as those described for streptomycin by Kuehl *et al.*[15] The concentrates used for the chromatograms illustrated in FIGURES 7 and 8 were of rather low activity. Comparison with FIGURES 3 and 5, for alumina and Darco G-60 carbon, clearly shows that, whereas the activity maximum appears before the solids maximum in the case of streptomycin, the opposite is true with streptothricin. The evidence indicates that the impurities in streptothricin move at a faster rate than the active substance. Since the pH of the concentrate solutions of both antibiotics were of about the same magnitude, streptothricin appears to be more strongly adsorbed than streptomycin.

Streptomycin and streptothricin are produced under very similar conditions by related microorganisms. It is reasonable to assume that, of the impurities present in crude concentrates, a considerable amount would

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[18] Peck, R. L., A. Walti, R. P. Graber, E. H. Flynn, C. E. Hoffhine, Jr., V. Allfrey, & K. Folkers. J. Am. Chem. Soc. 68: 772. 1946.

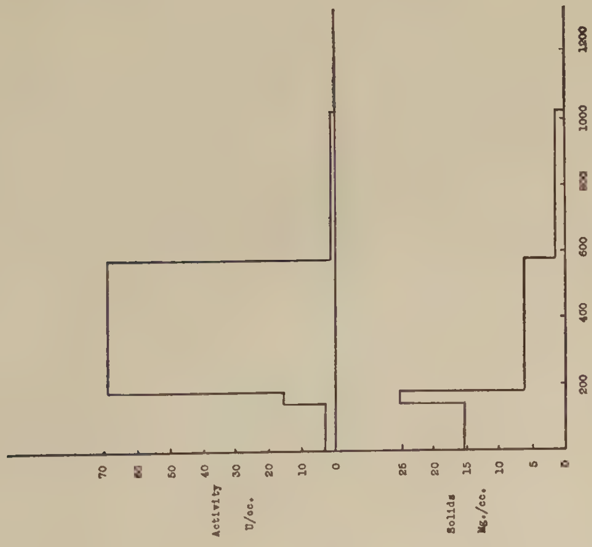

FIGURE 7. Chromatogram of streptothricin hydrochloride, 49 units/mg. Solvent: methanol; adsorbent: alumina. (Data from **Peck et al.**[18])

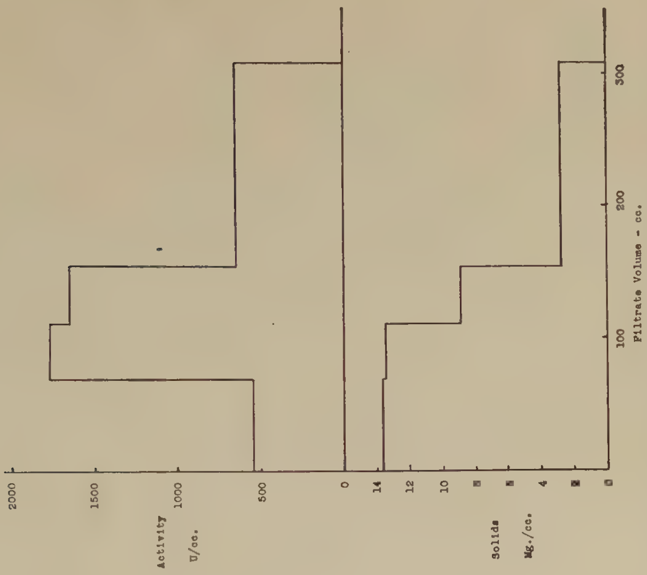

FIGURE 8. Chromatogram of streptothricin hydrochloride, 120 units/mg. Solvent: methanol; adsorbent: Darco G-60. (Data from **Peck et al.**[18])

be the same for equivalent concentrates of the two antibiotics. On the basis of this assumption, and from the data compared above, it would be anticipated that a mixture of streptomycin and streptothricin could be separated chromatographically by the systems described above and that the streptomycin would appear first in the filtrate fractions.

When it was found that the most active fractions obtained by chromatography of streptomycin hydrochloride in methanol on alumina or Darco G-60 were in the first filtrate fractions collected, it was apparent that streptomycin was about the least strongly adsorbed component of crude concentrates. The conclusion was drawn[15] that, by adding portions of alumina to a methanolic solution of streptomycin hydrochloride, a substantial amount of the impurities present would be removed, thus effecting an increase in the activity of the streptomycin remaining in solution. This proved to be true, although the efficiency of this purification method was somewhat less than that of the chromatographic procedure.

Mueller[19] recently described a moderately large-scale application of this method, employing streptomycin phosphate-hydrochloride in absolute methanol. Washed Harshaw alumina (passing 80 mesh) was used as adsorbent. Streptomycin preparations of 200-300 units per mg. could be increased in activity to any desired degree up to a practical limit of 650 units per mg. Adsorption isotherms for two crude streptomycin salts were determined by Mueller[19] for various conditions. Curves showing activities, together with total recovered solids and activities, permit the yield and activity to be predicted for the product of any operation. The method is useful where the highest activities are not desired.

## SUMMARY

The chromatographic purification of streptomycin has been conducted with acid-washed alumina and with Darco G-60 carbon as adsorbents and with methanol or 80 per cent methanol as developing solvents. Concentrations employed varied over a wide range. An example of fairly large-scale chromatography of a concentrate has been described. Streptomycin appears to pass down chromatographic columns at a rate faster than that of most of the other components of the crude concentrates used, that is, it was least strongly adsorbed. The rate does, however, appear to depend somewhat on the pH of the solutions employed, and is slower at the higher pH values. Batch treatment of methanol solutions of streptomycin with alumina removed most of the impurities and left the active material in solution in a form readily isolated.

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[19] **Mueller, G. P.** Abstracts of the Chicago Meeting, A. C. S., Sept., 1946: 14B.

# PARTITION CHROMATOGRAPHY

*By* A. J. P. Martin

*Boots Pure Drug Company Limited, Nottingham, England*

Synge,[1] in 1938, was preparing the acetyl derivatives of the amino acids and showed that there were considerable differences between the partition coefficients of many of them between $CHCl_3$ and water. He felt that these differences could be made the basis of a scheme of separation of the more fatty mono-aminomonocarboxylic acids. Martin, some time previously, had constructed a counter-current extraction machine for vitamin purification. This machine, though at its best it had an efficiency of some 200 theoretical plates, was unsuitable for the chloroform-water system needed for the acetyl amino acids. Martin and Synge,[2] therefore, constructed a different type of machine with an efficiency of less than 40 theoretical plates, which was used for the separation of certain of the amino acids in a hydrolysate of wool. However, the quantity of 40 theoretical plates was inadequate to obtain clean-cut fractions. The mechanical and running difficulties, at the same time, precluded the use of a machine of much higher efficiency, and other methods were tried.

The essential trouble with the machines was that, if the size of the drops of the dispersed phase was small enough to permit equilibrium to be attained in a reasonable time, then it was difficult to obtain the required relative movement of the two phases, *i.e.*, entrainment occurred or the settling time was excessive.

An attempt was made to overcome this trouble by using a column packed with parallel fibers of wool and cotton, with the idea that the cotton would wet with the aqueous phase and the wool with the organic phase, and that many parallel counter-current streams could be maintained in a small tube. Not surprisingly, perhaps, this scheme did not work and large areas became the domain of one phase only.

The idea of obtaining flow in both directions was therefore abandoned, and an attempt was made to hold one phase stationary while the other was allowed to flow past it.

Silica gel was chosen to hold water stationary while chloroform flowed past it. The silica would hold about half its weight of water without apparently becoming wet, and chloroform did not tend to displace the water. It was possible, therefore, to make up a "partition chromatogram" with silica gel containing water and, using chloroform as the mobile phase, to effect a separation between various acetyl amino acids, making use of their difference in partition coefficients between chloroform and water. It was, of course, necessary that the silica itself should not adsorb too much of the acetyl amino acids and we were fortunate in that, for the first experiment, a commercial chloroform was used which contained appreciable amounts of ethyl alcohol. In a second experiment using purified chloroform, the acetyl amino acids were all held at the top of the column, and it was found, later, that some relatively polar compound had to be present in the solvent to act as an eluting agent, to prevent the silica, as far as possible, from acting as an adsorbent.

There were, of course, several materials which could have been used to hold water in this way. Silica, however, had one particular advantage. The acetyl amino acids being quite strong acids, it was feasible to reveal what part of the column they occupied by including, in the water in the silica, an indicator such as methyl orange. The column then turned to red (from yellow or orange) in the regions where the acetyl amino acids were present. It was not possible to make this indicator-color change satisfactory in any other water-holding substance, the range of the indicator usually being shifted to a lower pH. The question of suitable indicators will be discussed below.

Since the problem of the separation of the acetyl amino acids had been approached from the angle of the counter-current extraction machines, it was natural to try to compare the respective efficiencies of the machines, as well as the partition chromatograms. A theory was therefore developed[3] expressing the efficiency of the chromatogram in terms of the number of theoretical plates of an extraction apparatus to which it was equivalent. For an average column, it was found to be of the order of thousands, this high efficiency being, of course, directly referable to the smallness of the distance that the solute had to diffuse to get into and out of either phase. It is, therefore, improbable for analytical purposes that any extraction machine can compete with the partition chromatogram, where the latter is suitable.

To return to indicators. One of the desirable characteristics of an indicator is insolubility in the mobile phase. In this respect, methyl orange is unsuitable with solvents containing high proportions of alcohol. For use with such solvents, Gordon, Martin, and Synge[4] introduced the use of the anthocyanins, peonin and pelargonin, while Liddell and Rydon[5] used 3:6-naphthalene-azo-N-phenylamine. These indicators, particularly the latter which is easily prepared, are satisfactory, being washed from the column slowly enough to permit the estimation of the required acetyl amino acids.

The indicator imposes, apart from questions of solubility, some limitation on the solvent systems that can be employed. With methyl orange, chloroform shifts the color towards yellow, and alcohols do so still more. Cyclohexane alone does not have much effect and, hence, larger proportions of alcohols have to be used with cyclohexane than with chloroform to obtain a usable color of the indicator. In general, alcohols of smaller molecular weight have relatively more effect on the color of the indicator and less effect on the partition coefficient of the acetyl amino acids than do alcohols of larger molecular weight.

From the theory given by Martin and Synge,[3] it is easy to show that if:

$A$ is the area of cross section of the column,

$A_S$ is the area of cross section of the non-mobile phase,

$A_L$ is the area of cross section of the mobile phase,

$A_I$ is the area of cross section of the inert solid $(A_S + A_L + A_I = A)$,

$\alpha$ is the partition coefficient, *i.e.*, $\dfrac{\text{g. solute/l. of non mobile phase}}{\text{g. solute/l. of mobile phase}}$,

$$R = \frac{\text{movement of position of maximum concentration of solute}}{\text{simultaneous movement of surface of developing phase in empty part of tube above chromatogram column}},$$

then, $R = \dfrac{A}{A_L + \alpha A_S}$ or $\alpha = \dfrac{A}{RA_S} - \dfrac{A_L}{A_S}$

The authors found[3] that, in the case of a particular column, the partition coefficient deduced from the rate of movement of the bands agreed very closely with that determined directly. This was taken to show that, under favorable conditions, adsorption could be practically eliminated, and that the water in the silica had the same solvent properties as normal water. In TABLE 1 are given the partition coefficients found for acetylproline and acetylphenylalanine, as well as those determined directly in the same solvent mixture. TABLE 2 shows the $R$ values for a number of acetyl amino acids and solvents.

TABLE 1

| Acetyl amino acid | $R$ | $\alpha$ determined from $R$ | $\alpha$ determined directly |
|---|---|---|---|
| Acetylproline | 0.37 | 9.4 | 9.5 |
| Acetylphenylalanine | 1.07 | 1.4 | 1.3 |

TABLE 2

BAND RATES OF ACETAMINO ACIDS WITH VARIOUS SOLVENTS

Figures are values of *R*

| Developing solvents | Butanol-chloroform | | | Propanol-cyclohexane | | Ethyl acetate |
|---|---|---|---|---|---|---|
| | 1% | 3% | 17% | 5% | 30% | |
| Acetylphenylalanine | 0.5 | 0.9 | Fast | 0.3 | Fast | Fast |
| Acetyl*nor*leucine | 0.4 | — | Fast | — | — | — |
| Acetylleucine<br>Acetyl*iso*leucine | 0.3 | 0.6 | Fast | 0.3 | Fast | Fast |
| Acetyltryptophane | 0.3 | 0.6 | Fast | 0.15 | Fast | — |
| Acetyl*nor*valine<br>Acetylmethionine | 0.15 | 0.3 | Fast | 0.15 | Fast | 0.9 |
| Acetylmethionine | 0.15 | 0.3 | Fast | 0.09 | 0.7 | 0.8 |
| Acetylproline | 0.15 | 0.3 | Fast | 0.04 | 0.3 | 0.2 |
| Acetaminobutyric acid | 0.07 | — | — | — | — | 0.5 |
| Acetylalanine | 0.025 | 0.04 | 0.35 | 0.04 | 0.3 | 0.2 |
| N-Acetyltyrosine | 0.02 | 0.04 | 0.7 | — | 0.6 | Fast |
| Acetylglycine | Slow | Slow | 0.15 | Slow | 0.15 | 0.1 |
| Acetylaspartic acid | | | | | | |
| Acetylglutamic acid | Slow | Slow | 0.1-0.2 | — | — | — |
| N,N Diacetylcystine | | | | | | |
| N,N Diacetyllysine | | | | | | |
| N,Acetylhydroxyproline | Slow | Slow | 0.07 | Slow | 0.1 | 0.06 |

The results given above have been shown to only one significant figure because, unless great care is taken in manufacture of the silica, moisture content, and in preparation of the column, the values are not consistent to better than this figure. The ratios are, however, much more consistent and, if several of the appropriate amino acids are present, there is never any difficulty in identifying a given zone; when only one zone is visible, it is desirable to confirm the identification by running chromatograms of the unknown mixed with an authentic sample of the substance that a preliminary run has indicated it to be. The question of the preparation of the silica will be taken up again below.

The procedure in analyzing a protein hydrolysate for the higher monoaminomonocarboxylic acids was as follows: The protein was hydrolyzed in a large excess of 6N HCl, boiling for 24 hours, and the HCl was largely removed by repeated distillation *in vacuo,* with additions of distilled water. The hydrolysate, corresponding to 25 mg. of protein, was then acetylated with five successive portions of $Ac_2O$ and NaOH, the mixture being maintained ice-cold and alkaline throughout. It was then made strongly alkaline for 10 minutes at room temperature, to hydrolyze any O-acetylation that might have occurred. Finally, it was acidified with 10 N $H_2SO_4$ and extracted, in a separatory funnel, with 17% BuOH-$CHCl_3$ mixture. (If acids slower moving than alanine are required—and this will be only if but one or two such acids are present—

complete extraction, which cannot conveniently be obtained in a separatory funnel, can be obtained by taking up the acetylation mixture in dry silica, making it into a column, and extracting with the BuOH-$CHCl_3$ mixture. A 1-cm. layer of water-saturated silica, introduced by Tristram,[6] is required below the main column to prevent any mineral acid from being extracted.)

The BuOH-$CHCl_3$ solution of acetyl amino acids was evaporated down and taken up in one or two ml. of 1% BuOH-$CHCl_3$. It was now ready to be applied to the column, which was prepared as follows: To 3 grams of dry silica in a 100-ml. beaker were added 1.5 ml. of a saturated solution of methyl orange, and made homogenous through light grinding with a test tube. 1% BuOH-$CHCl_3$ was added until a cream was produced, and this was then poured into the chromatogram tube. When the column had settled and drained, no entry of air into the column occurred, provided that the lower end of the tube was not allowed to become quite dry. Acetyl amino acid solution was then run carefully onto the column and allowed to soak in, and followed by three rinsings with similar volumes. The top of the column had now changed in color from orange-pink to bright red. The column was then developed with 1% BuOH-$CHCl_3$ mixture, and zones of acetylphenylalanine, acetylleucine + acetyl*iso*leucine, acetylproline + acetylvaline + acetylmethionine, and acetylalanine could be distinguished, with the other acetyl amino acids left at the top of the column. The first three of these zones were run out and collected separately. The point at which a cut should be made, could be determined by estimating, while zones on either side of the proposed cut were still visible, the amount of solvent standing above the column which should run in, so that the cut be made at the desired $R$ value. With 1% BuOH-$CHCl_3$, an excessive volume would have been required to run out the acetylalanine zone, so a change was made to 17% BuOH-$CHCl_3$. Acetyltyrosine then overtook the acetylalanine, and both zones were collected separately.

After evaporation of the solvent, the fraction containing acetylproline, acetylmethionine, and acetylvaline was run on a column using 5% PrOH-$C_6H_{12}$. Acetylvaline and acetylmethionine were then collected separately, and acetylproline was run out by changing the solvent to 30% PrOH-$C_6H_{12}$. It was also found advisable to put the other zones from the $CHCl_3$ column through the cyclohexane column, which separated out some non-acetyl amino acid impurities. Finally, after evaporation of the solvents, the individual fractions were titrated against $\frac{N}{100}$ $Ba(OH)_2$ solution, using bromophenol blue as indicator.

In this way, quantitive analyses of 25 mg. of protein hydrolysate for phenylalanine, leucine and *iso*leucine, valine, methionine, proline, tyrosine, and alanine were obtained. The order in which the various solvents were used may be changed in certain cases (see reference 6).

It was found, from analyses of control mixtures of amino acids treated exactly as the protein, that results within 5 per cent of true values were obtained. It was thus considered desirable always to compare results obtained with a given protein with those obtained from a control mixture made up to simulate as closely as was possible the protein under investigation. Correction factors, derived from the results of the analysis of the control mixture, were then applied to the results of the protein analysis, and some further improvement of accuracy probably resulted.

All the sources of error have not been discovered. It was found that results obtained from different acetylations differed more than those obtained from the same acetylation, and that much better results could be obtained from mixtures of pure acetyl amino acids. Apart from failure to attain complete acetylation, there is a possibility of peptide synthesis as a consequence of the formation of a mixed anhydride, by reaction between acetic anhydride and the carboxyl of the amino acid, or acetyl amino acid.

There is also evidence of the presence, in the acetylated hydrolysate, of acids containing less nitrogen than the acetyl amino acids and, presumably, derived from decomposition products of the protein.

Tristram[6] has also found great variation in the recovery of methionine, due, probably, to oxidation of the sulphur. A discussion of his results in an extensive series of analyses is given in the same paper.

However, probably the biggest source of error lies in the silica. It has not been found possible to devise a method of consistently preparing satisfactory silica, though, recently, new methods have been proposed of which the author has no experience. The silica may fail either in that the color of the indicator is such that the acetyl amino acid zones are unobservable, or in its absorbing the acetyl amino acid with the result that a zone of one acetyl amino acid tails into the succeeding zone. Tests (Gordon, Martin and Synge,[4] Tristram[6]) involving separations of test mixtures have been proposed for excluding unsatisfactory batches. Different batches of water glass used for the preparations of the silica require different methods, and further work on this subject is needed. Isherwood[7] believes that many of the troubles are due to the presence of iron and, in his method, takes particular pains to remove this.

The partition chromatogram on silica has been used for the estimation of a number of other acids Hanby and Rydon[8] have converted glutamic acid to pyrrolidone carboxylic acid by autoclaving at 100° for 4 hours in a solution brought to pH 2 with acetic acid. The pyrrolidone carboxylic acid can then be run with 17% BuOH-$CHCl_3$ ($R = 0.3$) and overall recoveries of more than 95 per cent are obtainable.

Elsden[9] has used the method for the smaller fatty acids, taking the method for 1 or 5% BuOH-$CHCl_3$ and bromocresol green as an indicator. The acids are titrated using a $CO_2$-stirred emulsion. TABLE 3 shows the recoveries he has obtained.

Table 3

| | Total acid (ml) | Butyric acid (ml) | Propionic acid (ml) | Acetic acid (ml) |
|---|---|---|---|---|
| *Theoretical* | 28.93 | 9.31 | 9.58 | 10.04 |
| *Observed* | 28.86 ± 0.35 | 9.4 ± 0.18 | 9.52 ± 0.26 | 9.93 ± 0.31 |

Patterson[10] has also used the silica partition for the separation of fatty acids, with satisfactory results. Sanger[11] has used silica columns with a variety of solvents for the separation of dinitro phenyl derivatives of amino acids. There is reason to believe, however, that, in his case, adsorption on the silica was playing a more important role than partition between the solvents and water. When silica suitable for use with the acetyl amino acids is employed, the *R* values are all much higher than those quoted by Sanger, and no useful separations are obtained. This is inexplicable if partition is the dominating phenomenon. Isherwood[7] has used the partition chromatogram on silica for the separation of a number of hydroxy acids from fruits. He has found it desirable to work with dilute sulphuric acid on the silica and, hence, cannot use an indicator in the column. He has found, however, that it is possible to run the effluent from the column down a short capillary tube with a slow stream of dilute alkali containing an indicator. The color of the indicator as it emerges from the capillary shows whether acid is running from the column. This method is potentially of very wide application, and its use with the acetyl amino acid separations might well avoid several of the snags associated with the use of silica. The accuracy of Isherwood's analyses for hydroxy amino acids is comparable with those of the acetyl amino acids.

Partition chromatograms have also, of course, been widely used for the separation and purification of the various penicillins. A buffer is used in the silica in place of water, and various solvents and buffers have been used. Most of the literature on this is not as yet published (see reference 12).

## PARTITION CHROMATOGRAPHY ON PAPER AND STARCH

Gordon, Martin, and Synge[13] were not able to separate the slower moving acetyl amino acids on silica and turned to the problem of the separation of the amino acids on partition columns. Following Dakin's work, and also that of England and Cohn, the first solvent tried was, of course, butyl alcohol. However, the silica was found to adsorb the amino acids too strongly, particularly those of higher molecular weight.

Of course, no indicator could be used for the amino acids and, hence, one of the chief reasons for preferring silica to other substances for holding the water was no longer valid.

The first choice among other possible materials was cellulose. Using a semicircle of filter paper as in the well-known technique of capillary analysis[14], but ensuring that the cellulose was saturated with water by carrying out the experiment in a closed box whose atmosphere was kept saturated with water vapor, it was found readily possible to separate various mixtures of amino acids, and the separation could be revealed after drying the paper by spraying with a solution of 0.1 per cent ninhydrin in BuOH and heating. The amino acids were then shown up as colored rings. Later, it was found more convenient to use strips of paper hanging from a trough containing the solvent saturated with water, the whole being enclosed in a vessel, the air within which was saturated with respect to both solvent and water. This work was continued by Consden, Gordon, and Martin.[15]

It was found, in the case of BuOH as solvent, that the rate of movement of the amino acid could be predicted from the amounts of water and solvent on the paper, and from the partition coefficient of the amino acid.

The relation of the partition coefficient to the rate of movement of the band may be calculated by the method of Martin and Synge.[3]

$A$ = cross-sectional area of paper + water + solvent,

$A_L$ = cross-sectional area of solvent phase,

$A_S$ = cross-sectional area of water phase,

$$\alpha = \text{partition coefficient}, = \frac{\text{concentration in water phase}}{\text{concentration in solvent phase}},$$

$$R = \frac{A}{A_L + \alpha A_S}$$

However, $R$ is not conveniently measurable in paper chromatograms, so that a new symbol, $R_F$ was introduced:

$$R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}} = \frac{RA_L}{A} = \frac{A_L}{A_L + \alpha A_S},$$

$$\text{or } \alpha = \frac{A_L}{R_F A_S} - \frac{A_L}{A_S} = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right).$$

$A_L/A_S$ is equal to the ratio of the volumes of solvent and water phase in the chromatogram.

Table 4

Partition Coefficients Calculated from $R_F$ Values

| No. of run | 1 | 2 | 3 | 4 | Direct measurements (England & Cohn*) |
|---|---|---|---|---|---|
| Per cent water in paper | 28.7 | 18.0 | 22.6 | 17.7 | |
| $A_L/A_S$ | 3.25 | 4.56 | 3.70 | 2.93 | |
| *Amino acid* | *Partition coefficients* | | | | |
| Glycine | 70.4 | 70.4 | 70.4 | 70.4 | 70.4 |
| Alanine | 35.9 | 39.9 | 43.7 | 36.6 | 42.3 |
| Valine | 12.2 | 14.1 | 14.8 | 12.5 | 13.8 |
| *Nor*valine | 8.7 | 10.8 | 10.5 | 9.2 | 9.5 |
| Leucine | 4.5 | 5.4 | 5.6 | 6.0 | 5.5 |
| *Nor*leucine | 3.5 | 4.2 | 4.4 | 4.6 | 3.2 |

* J. Am. Chem. Soc. 57: 634. 1935.

Assuming a given water content of the paper, $A_L/A_S$ may be deduced from the ratio of weight of dry paper to that of the developed chromatogram.

The water content of the paper is apt to vary from experiment to experiment and is difficult to measure in the presence of *n*-butanol or another solvent. TABLE 4 shows the partition coefficient calculated from the $R_F$ and $A_L/A_S$ values for four separate runs under slightly different conditions. The water content has been assumed so that the partition coefficient for glycine is equal to that given by England and Cohn (1935). The last column gives the direct measurements of England and Cohn.

The water content of saturated cellulose according to the *International Critical Tables,* is 22 per cent on a dry-weight basis.

TABLE 5 shows a number of $R_F$ values for various amino acids and the most commonly used solvents. Some additions have been made in some solvents, *e.g.,* cupron, HCN, and coal gas. These were to overcome a puzzling and unexpected difficulty. In many of the early chromatograms run with phenol, butyl alcohol, and benzylalcohol, a purple spot showing the amino acid was preceded by a pink "beard" of rather faster-running material, and, in general, it was found that the further the amino acid had run, the larger was the pink spot and the smaller the purple one. Thus, by the time leucine had run to the bottom of the sheet, it might be almost completely converted to a rather ill-defined pink streak. This was found to be due to the combination of the amino acid with copper which was in the paper, the copper complex having a higher $R_F$ value than the corresponding amino acid. This trouble could be overcome by including in the atmosphere or solvent some substance that would form a more stable complex with the copper than the amino acid copper complex. It was found later[16] that, in the case of cysteic acid, zinc also formed a complex, though this was slower-running than the amino acid.

In TABLE 5, it will be noticed that the $R_F$ values in collidine of an amino acid and the corresponding hydroxy amino acid are very similar, whereas the $R_F$ values in phenol are considerably depressed by the addition of a hydroxyl group to the molecule. This indicates that little energy is required to transfer a hydroxyl group from water to collidine, but relatively much from water to phenol. A comparison of aromatic and aliphatic amino acids in butanol and benzyl alcohol shows similar differences. The effect of including $NH_3$ in the atmosphere on the $R_F$ values of the basic and acidic amino acids also follows the expected pattern. It is hoped that, as further experience with different solutes and solvents is obtained and information about energies of association of various groups increases, a rational approach will make it possible to select the most effective solvent for a given separation. It may be that the partition chromatogram will, in this way, prove to be more predictable and even more versatile than the adsorption chromatogram.

It will be seen in TABLE 5 that the order of the various acids changes in different solvents. This suggested the possibility of further useful

TABLE 5

$R_F$ VALUES OF AMINO ACID IN VARIOUS SOLVENTS ON WHATMAN NO. 1 PAPER, AT ROOM TEMPERATURE

| Solvent | Phenol | Phenol | *s*-Collidine | *n*-Butanol | Benzyl alcohol | *o*-Cresol | *o*-Cresol | *m*-Cresol | *m*-Cresol | *p*-Cresol | *p*-Cresol | *Iso* butyric acid |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Addition | 0.1% $NH_3$* coal gas | Coal gas | — | Cupron | HCN | Cupron | Cupron 0.1% $NH_3$* | Cupron | Cupron 0.1% $NH_3$* | Cupron | Cupron 0.1% $NH_3$* | — |
| Glycine | 0.41 RP | 0.40 | 0.25 P | 0.05 | 0.02 | 0.07 | 0.07 | 0.12 P | 0.14 | 0.15 RP | 0.19 | 0.36 |
| Alanine | 0.55 P | 0.57 | 0.32 P | 0.08 | 0.03 | 0.13 | 0.15 | 0.23 P | 0.24 | 0.28 | 0.32 | 0.44 |
| *Nor*valine | 0.78 P | 0.78 | 0.48 P | 0.26 | 0.12 | 0.45 | 0.45 | 0.60 B | 0.62 | 0.66 BP | 0.66 | 0.71 |
| Valine | 0.76 P | 0.78 | 0.45 P | 0.20 | 0.11 | 0.41 | 0.38 | 0.52 P | 0.54 | 0.59 BP | 0.60 | 0.65 |
| *Nor*leucine | 0.87 P | 0.85 | 0.60 P | 0.47 | 0.27 | 0.72 | 0.67 | 0.80 B | 0.78 | 0.82 BP | 0.80 | 0.79 |
| *Iso*leucine | 0.87 P | 0.82 | 0.54 P | 0.37 | 0.18 | 0.58 | 0.55 | 0.70 B | 0.70 | 0.73 BP | 0.74 | 0.76 |
| Leucine | 0.86 P | 0.84 | 0.58 P | 0.38 | 0.21 | 0.61 | 0.62 | 0.73 B | 0.73 | 0.73 BP | 0.77 | 0.78 |
| Phenylalanine | 0.90 P | 0.86 | 0.59 G | 0.43 | 0.36 | 0.81 | 0.74 | 0.82 GB | 0.82 | 0.82 P | 0.83 | 0.80 |
| Tyrosine‡ | 0.66 GP | 0.59 | 0.64 G | 0.28 | 0.14 | 0.24 | 0.23 | 0.35 P | 0.35 | 0.39 B | 0.38 | 0.58 |
| Serine | 0.35 P | 0.33 | 0.28 G | 0.05 | 0.01 | 0.05 | 0.05 | 0.08 BP | 0.08 | 0.11 P | 0.13 | 0.34 |
| Threonine | 0.47 P | 0.50 | 0.32 P | 0.07 | 0.02 | 0.11 | 0.08 | 0.14 B | 0.14 | 0.19 P | 0.21 | 0.43 |
| Hydroxyproline | 0.66 O | 0.66 | 0.34 GY | 0.07 | 0.04 | 0.27 | 0.23 | 0.32 GY | 0.32 | 0.40 BG | 0.42 | 0.42 |
| Proline | 0.89 Y | 0.87 | 0.35 Y | 0.12 | 0.12 | 0.69 | 0.66 | 0.73 Y | 0.75 | 0.76 Y | 0.78 | 0.57 |
| Tryptophane | 0.80 P | 0.76 | 0.62 P | 0.35 | — | 0.65 | 0.58 | 0.76 | 0.69 | 0.70 B | 0.68 | — |
| Histidine‡ | 0.70 RP | 0.72 | 0.28 G | 0.06 | 0.02 | 0.25 | 0.33 | 0.34 P | 0.44 | 0.36 B | 0.52 | 0.45 |
| Arginine | 0.85 P | 0.67 | 0.16 P | 0.03 | 0.01 | 0.02 | 0.44 | 0.07 P | 0.76 | 0.12 BP | 0.81 | 0.40 |
| Ornithine‡ | 0.61 P | 0.40 | 0.13 BG | 0.01 | 0.00 | 0.01 | 0.21 | 0.03 P | 0.47 | 0.04 P | 0.59 | 0.24 |
| Lysine‡ | 0.73 P | 0.50 | 0.14 BG | 0.01 | 0.00 | 0.01 | 0.20 | 0.04 P | 0.54 | 0.07 BP | 0.66 | 0.27 |
| Aspartic acid** | 0.12 BP | 0.14 | 0.22 B | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 RP | 0.01 | 0.02 BP | 0.02 | 0.31 |
| Glutamic acid | 0.19 P | 0.24 | 0.25 P | 0.01 | 0.00 | 0.01 | 0.01 | 0.01 RP | 0.02 | 0.03 BP | 0.03 | 0.38 |
| Lanthionine‡ | 0.21 RP | 0.18 | 0.12 G | 0.01 | 0.00 | 0.00 | 0.01 | 0.01 R | 0.03 | 0.03 RP | 0.05 | 0.21 |
| Cystine‡ | 0.13 RP | 0.13§ | 0.14 G | 0.01 | 0.00 | 0.04 | 0.01 | 0.04 P | 0.04 | 0.02 RP | 0.02 | 0.25 |
| Methionine | 0.83 P | 0.82 | 0.57 GP | 0.26 | 0.17 | 0.58 | 0.49 | 0.64 B | 0.63 | 0.71 BP | 0.68 | 0.69 |

Color given by ninhydrin: B, Blue; G, Grey; O, Orange; P, Purple; R, Red; Y, Yellow.

* The % figure refers to the strength of $NH_3$ solution present in quantity in the tray.

‡ These acids were put on as the hydrochlorides and neutralized with $NH_3$ before development.

§ Cystine is decomposed by these additions. $R_F$ value refers to the decomposition product.

** Color initially green in presence of phenol collidine. Only final color shown in table.

separation by two runs at right angles with different solvents, thus producing a two-dimensional chromatogram.

A sheet of filter paper (Whatman No. 4) 18 by 22″ is taken and near one corner a drop of protein hydrolysate is placed. The sheet is then hung from the trough and run with *s*-collidine. After a sufficient length of time (12 to 36 hours, depending on kind of paper and solvent), the paper is dried, turned through a right angle, and again run with phenol (in an atmosphere of coal gas with some $NH_3$ present). It is again dried, sprayed with ninhydrin solution and heated. Colored spots appear, showing the positions of the various amino acids. With the pair of solvents mentioned, cystine, cysteic acid, aspartic acid, glutamic acid, serine, glycine, threonine, alanine, hydroxy proline, proline, hydroxylysine, lysine, arginine, histidine, valine, and tyrosine appear as separate spots, while *iso*leucine, leucine, methionine, and phenylalanine and tryptophane fall together in an elongated spot. By using benzyl alcohol and butyl alcohol as the two solvents, those acids which are not separated by the collidine phenol chromatogram can be separated, though other amino acids will not then be resolved. The amount of protein hydrolysate required for one such two-dimensional chromatogram is about 300 micrograms. The amount of an individual amino acid that can be seen is 1 to 2 micrograms, and on a one-dimensional strip, half a microgram can, under favorable circumstances, be observed. One of these two-dimensional chromatograms is illustrated and interpreted in FIGURE 1.

In many instances, of course, the small quantity required for an analysis is a great advantage. Thus, it is possible to get much information from small amounts of valuable products, and to control fractionations by other methods, while using negligible aliquots. It is obvious that this method can be used in testing for other amino acids in allegedly pure amino acids.

The method is well adapted to any analysis for amino acids in which qualitative information is all that is required. Thus, Consden, Gordon, Martin, Rosenheim, and Synge[16] examined the samples of Thudichum's "glycoleucine" which had been isolated by him in 1880 from brain tissue and whose nature was uncertain, but which had come in many quarters to be regarded as *nor*leucine. Running comparative chromatograms of glycoleucine, *nor*leucine, leucine, and various mixtures of these, it was rapidly ascertained that glycoleucine was leucine. Other methods confirmed this and showed it to be *dl*-leucine (since it was from a baryta hydrolysate, this was, of course, only to be expected in the light of knowledge not available in 1880). A search was then made for *nor*leucine in hydrolyzed spinal cord, by the method of running the hydrolysate alone, *nor*leucine alone, and mixtures of the two, using benzyl alcohol as the solvent. It was shown that not more than .03 per cent of the nitrogen could be present as *nor*leucine, since when ½ microgram of *nor*leucine was added to 1.2 mg. of hydrolysate it could be clearly recognized, but none could be detected in the hydrolysate alone.

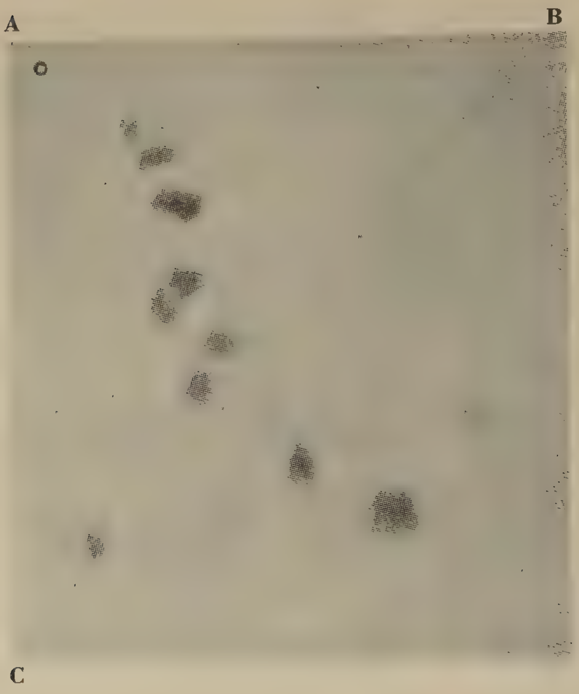
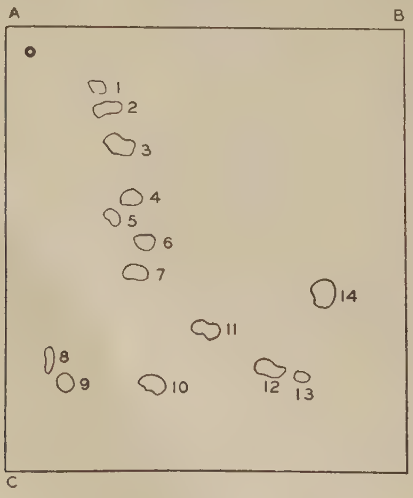


FIGURE 1. Two-dimensional chromatogram of a wool hydrolysate (180 μg.) on Whatman No. 1 sheet. Hydrolysate applied at circle. Run with collidine for 3 days in direction *AB*, then in direction *AC* with phenol for 27 hours in an atmosphere of coal gas and $NH_3$ (produced from a 0.3 per cent $NH_3$ solution). The filter employed in photographing renders the yellow proline spot scarcely visible. (From Biochem, J. 38, Plate 1, opp. p. 226. 1944.)



1 = Cystine
2 = Aspartic acid
3 = Glutamic acid
4 = Serine
5 = Glycine
6 = Threonine
7 = Alanine
8 = Lysine
9 = Arginine
10 = Proline
11 = Valine
12 = Leucine
Isoleucine
Methionine
13 = Phenylalanine
14 = Tyrosine

The paper partition chromatogram has been applied by Dent[17] to an extensive investigation of pathological urine, particularly in Fanconi's syndrome. The method has also been used by Edman[28] for the investigation of hypertension.

Partridge[18] has adapted the method to the investigation of the reducing carbohydrates. The same solvents that are useful for the amino acids are, surprisingly, useful also for the sugars. Instead of ninhydrin, Partridge sprays the paper with ammoniacal silver nitrate. The unreduced silver is then washed away, and a brown stain is left where the carbohydrate had been. Chargaff[19] has applied the method to the analysis of nucleotides, and Fink[20] to the separation of fatty acids after conversion to the corresponding hydroxamic acids.

Elsden and Synge suggested the use of potato starch for the separation of amino acids. In view of the development of this method by Moore and Stein, described in this publication, it is inappropriate to give details of this work. Synge[20] has also used it for the fractionation of partial hydrolysis mixture of gramacidin.

A review with color photographs of column, and paper strip and sheet chromatograms has recently been published[29] and the literature to 1944 has been reviewed.[30]

## IDENTIFICATION OF SIMPLE PEPTIDES

Consden, Gordon, and Martin[21] found that simple peptides will, in general, run on the chromatogram as satisfactorily as amino acids. However, both because of their large number and also because the $R_F$ values are much more sensitive to slight changes in solvents, etc., it was not practicable to identify them by their $R_F$ values alone. The authors therefore developed the technique of washing them off the chromatogram and hydrolyzing, and deaminating and hydrolyzing them, and then identifying the products on other chromatograms.

If the mixture was initially somewhat complex, duplicate two-dimensional chromatograms were run and one of these was treated with ninhydrin in the usual way. This reveals the full picture. The other was treated with about one-tenth the usual amount of ninhydrin, a quantity insufficient to destroy more than a small fraction of the peptide but enough to give an indication of the position of the stronger spots. The positions of the weaker spots shown on the normally treated sheet could usually be interpolated from the stronger spots. The pieces were cut out in this form , and the flat end was applied to wet filter paper overhanging a water-filled trough. To the dependent point of the piece cut from the chromatogram, a capillary tube was adjusted. In the course of about an hour, 50-100 microliters ran into the capillary and this was found to be sufficient to wash about 10 cm.$^2$ of paper.

The liquid in the capillary was then transferred to a strip of polythene on which it formed a drop with no tendency to spread. The water was removed in a vacuum desiccator and a drop of 6N HCl added, the HCl solution was taken back into the capillary, the ends sealed, and put in an oven at 100°C overnight for hydrolysis. After removal of the HCl in the desiccator, again on a polythene strip, the amino acids were transferred to a paper chromatogram with water. If the peptide was to be deaminated, this was done by exposing it in 6N HCl on polythene to nitrous fumes.

Using this technique, Consden, Gordon, Martin, and Synge[22] studied the partial HCl hydrolysis products of gramicidin *S*. This has been shown by Synge[23] to consist of equimolecular proportions of the five amino acids, valine, ornithine, leucine, phenylalanine, and proline, and to be a cyclopeptide since there was only one free amino group and no free carboxyl group. The free amino group was shown by Sanger[24] to be the side chain amino of the ornithine. Among the partial hydrolysis products were identified valylornithine, ornithyl leucine, leucyl phenylalanine, phenylalanyl proline, and two tripeptides (not rigorously identified) phenylalanyl prolylvaline, and valyornithyl leucine.

Control chromatograms made up with the five amino acids and synthetic valylornithine, ornithyl leucine, leucyl phenylalanine, phenylalanyl proline, and prolyl valine gave a picture expected from that of the partial hydrolysis mixture, with the addition of the prolyl valine spot, and the synthetic mixture added to the partial hydrolysate showed only the expected number of spots. Prolyl valine proved to be more readily hydrolyzable than the other peptides, but this may not be the full explanation of the failure to observe it. These findings together with x-ray data from Dr. Crowfoot, consistent with Gramicidin-*S* being either a cyclopentapeptide or a cyclodecapeptide with a center of symmetry, show that the molecule consists of the amino acids, valine, ornithine, leucine, phenylalanine, and proline, united in that sequence once, or twice repeated, in a ring.

Consden, Gordon, and Martin[25,26] have examined hydrolysates of wool in a similar manner. The mixture was so complicated that little could be done unless separation by other means preceded chromatography. They therefore used ionophoresis as a first step and examined the fractions thus obtained chromatographically. Confirmation of their findings has to await synthesis of the many peptides encountered. Of particular interest, however, is the finding of several peptides of two united polar amino acids, and of two united non-polar amino acids, in contradiction to Astbury's hypothesis of the structure of keratin.

## QUANTITATIVE ANALYSIS OF AMINO ACIDS BY PAPER CHROMATOGRAPHY

Martin and Mittelmann[27] attempted, by several methods, to render the paper chromatograms quantitative. The great stumbling block is, of course, the small quantities that can be handled, and presence on the paper of not negligible quantities of soluble materials other than the amino acids.

The method they found most promising was a modification of Pope and Stevens's method of forming the copper complex of the amino acid and then estimating the copper in this. So far, only simple mixtures of amino acids have been analyzed which could be separated in a one-dimensional chromatogram. The technique was as follows: On a wide sheet of paper, well-separated spots of a control mixture and the mixture to be analyzed were alternately placed and the chromatogram developed. The chromatograms at either edge of the sheet were cut off and developed with ninhydrin, and then used as guides for cutting up the rest of the sheet. The pieces containing the separated amino acids were then, after cutting into small pieces, stood in a suspension of copper phosphate in a borax phosphate mixture, in centrifuge tubes. After standing overnight in an icebox, the copper phosphate and paper were centrifuged down and the copper in the supernatant liquid estimated by the polargraph, after the addition of sodium sulphite. The amounts of the control mixture put on the paper were graded so that the best straight line could be drawn, on a graph showing current/concentration for each amino acid. From these graphs, the amounts present in the unknown could be inferred.

So far, the only natural product analyzed has been germicidin-*S*. Synge has shown this to contain equimolecular proportions of the five amino acids. The amounts found by Martin and Mittelmann are shown in TABLE 6. Though the accuracy of these results is less than that obtainable by the silica method using acetyl amino acids, and less also than that obtained by the best of the microbiological methods, less than one milligram of material was consumed in the whole analysis. Work on this method is at present interrupted, and it is too early, as yet, to decide upon its final usefulness.

TABLE 6

| Amino acid | Solvent | Amount in each spot $10^{-7}$ mols. |
|---|---|---|
| Valine | *S*-Collidine | 1.65 |
| Proline | | 1.62 |
| Ornithine | | 1.55 |
| Leucine<br>Phenylalanine | Benzylalcohol | 1.53 |
| *Amount expected on Synge's formula:* 1.64 × $10^{-7}$ mols. | | 1.67 |

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# PARTITION CHROMATOGRAPHY OF AMINO ACIDS ON STARCH

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In the preceding paper of this conference, Dr. A. J. P. Martin[1] has described the investigations carried out in England which have led to the development of the technique known as partition chromatography. Our own experiments in this field resulted from an interest in the isolation and identification of amino acids and peptides in biological material. The recently developed principles of partition chromatography appeared to offer marked potentialities for this type of problem.

In considering the form of partition chromatogram most suitable for isolation work with amino acids and peptides, we were impressed by the demonstration, by Elsden and Synge,[2, 3] that raw potato starch was a satisfactory supporting medium for the aqueous phase. As the authors showed, starch columns possess the advantage that they can be applied directly to the fractionation of mixtures of free amino acids and peptides. The preliminary acetylation procedure required in the case of the silica gel columns, with its attendant drawbacks, is thus avoided. The same advantages indicated for starch hold also for the paper strip chromatogram.[4] Paper strips, however, handle only microgram quantities of material, whereas columns handle milligram quantities. From a column, therefore, sufficient material may be obtained to permit further investigation by conventional microchemical techniques.

In 1944, Synge[3] employed starch columns in experiments with hydrolysates of gramicidin. *n*-Butanol-water was used as the solvent system. The use of free amino acids and peptides, a major proportion of which are neutral substances, meant that visualization of the separation process as it occurred on the column could not be effected by means of a pH indicator, as it could be in the case of the fractionation of the acetylated amino acids on silica gel columns. The positions of amino acids after development of the starch column with butanol could be ascertained, however, by passing through a solution of ninhydrin in ether. From the positions of the resulting blue bands, the rates of movement of the amino acids on the column could be determined. For the fractionation of the unknown constituents of a partial hydrolysate of gramicidin, Synge collected the effluent in a series of fractions and tested each one qualitatively with ninhydrin paper. The volume of each fraction was adjusted in order to collect substances within a given range of distribution coefficients. On working up the fractions individually, several of the components of the partial hydrolysate were isolated and identified.

In adapting the starch column to our problems, we have made two additions to this procedure. Instead of collecting the effluent from the chromatogram in large fractions, the effluent has been divided into a regular series of very small fractions of known volume. These fractions have been analyzed quantitatively rather than qualitatively. The data thus obtained permit the construction of effluent concentration curves which reveal the detailed behavior and the full resolving power of the column.

A typical effluent concentration curve, showing the separation of phenylalanine, leucine, and isoleucine is illustrated in FIGURE 1. The advantages to be gained from employing precise curves of this type to visualize the results, have been well demonstrated in the counter-current distribution studies of Dr. Lyman C. Craig.[5, 6, 7] It is a pleasure to acknowledge many helpful and encouraging discussions with Dr. Craig on the subject of these experiments.

In the experiment referred to in FIGURE 1, the solvent was 1:1 *n*-butanol:benzyl alcohol nearly saturated with water. The effluent was collected in 1-cc. fractions. The amino acid concentration is expressed in millimoles per liter. The manual collection of 1-cc. fractions is obviously too tedious an occupation. The performance of a large number of experiments of this type became a practical laboratory procedure only after the construction of a fully automatic fraction collector,[8] which is illustrated in FIGURE 2. The column, with a solvent reservoir, is mounted over a circular rack containing eighty test tubes. The sample of amino acids, or amino acid hydrochlorides, dissolved in one or two cc. of the organic phase, has been added previously to the top of the column, drained into the starch, and the reservoir filled with solvent. Columns of the size shown

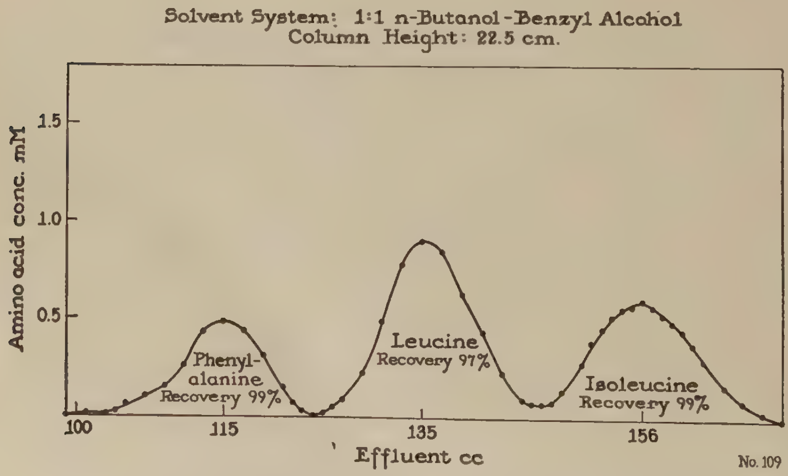

FIGURE 1. Separation of phenylalanine, leucine, and isoleucine.

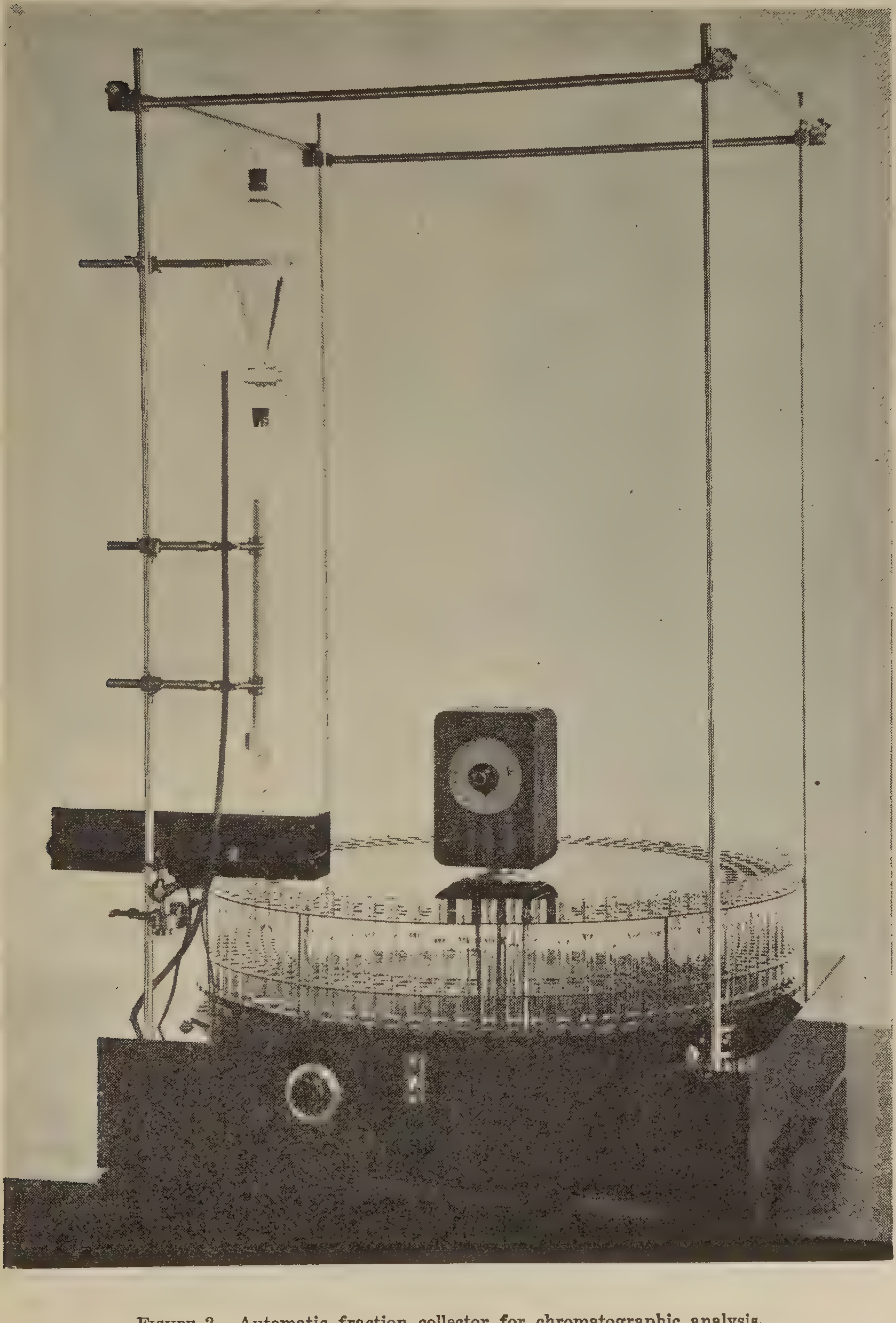

FIGURE 2. Automatic fraction collector for chromatographic analysis.

in FIGURE 2, about 2 cm. in diameter, are operated at a flow rate of about 5 cc. per hour, maintained either by gravity or by slight air pressure. As each drop emerges from the column, it intercepts a light beam focused on a photocell. The drops registered by the photocell are counted by an automatic-reset impulse counter. After a preset number of drops, corresponding to any desired volume from one drop to about 20 cc., the impulse counter resets itself to zero and closes the switch on a motor. The motor moves the rack 1/80 of a turn, bringing a new receiver under the column. The entire operation then starts over. A small funnel with a capillary tip blocks the mouth of each receiver, thus preventing evaporation. In practice, an individual experiment may run from 48 to 72 hours. The fraction collector permits day and night operation.

The next step involves the analysis of the numerous fractions collected. For this purpose, a quantitative colorimetric ninhydrin method has been developed.[9] Prior to quantitative analysis, however, the fractions are spot-tested on paper impregnated with ninhydrin. On the basis of the spot tests, the appropriate fractions can be chosen for quantitative analysis. For the determinations, small aliquots of the effluent fractions are pipetted into colorimeter tubes. A pipetting machine delivers, in the proper amount, the reagent which is stored under nitrogen in a reservoir attached to the machine. The colorimeter tubes are heated for 20 minutes in a boiling water bath, diluted to volume with a second pipetting machine, and read on a spectrophotometer. With this procedure, five hundred to one thousand analyses per week can be run conveniently by one individual.

The modified ninhydrin method gives reproducible results with most of the amino acids and with many peptides possessing a free $\alpha$-amino group. The principal disadvantage of the method is that the amino acids do not all give the same intensity of color per mole. To the extent that the chromatogram accomplishes separation of the individual amino acids from one another, the colorimetric method is capable of yielding quantitative values for any given amino acid.

Another disadvantage of the ninhydrin method is that it determines only compounds possessing amino groups. Continuous recording refractometry would have more general applicability. The sensitivity of the colorimetric ninhydrin method, however, which is 1 ppm. of amino acid in aqueous or alcoholic solution, is about 150 times that of refractometric measurements with an accuracy of $2 \times 10^{-5}\ n_D$. The combination of simplicity and sensitivity in the analytical method has been of importance in the experiments with partition chromatograms. Although the starch columns can handle milligram quantities, initially in fairly concentrated solution, the concentrations of components in the effluent progressively decrease as the fractionation process is extended to larger effluent volumes.

In returning to a consideration of FIGURE 1, it will be noted that, for cases where the procedure has been well worked out, the integration of the curves gives quantitative recovery for the components. Before a curve as satisfactory as this one could be obtained, many variables had to be brought under control. An early experiment with the same three amino acids and the same solvent system is shown in FIGURE 3. The irregularity and poor resolution in this case may be ascribed to several causes, among which are poor packing of the column, too rapid a flow rate, and suspended water droplets in the organic solvent.

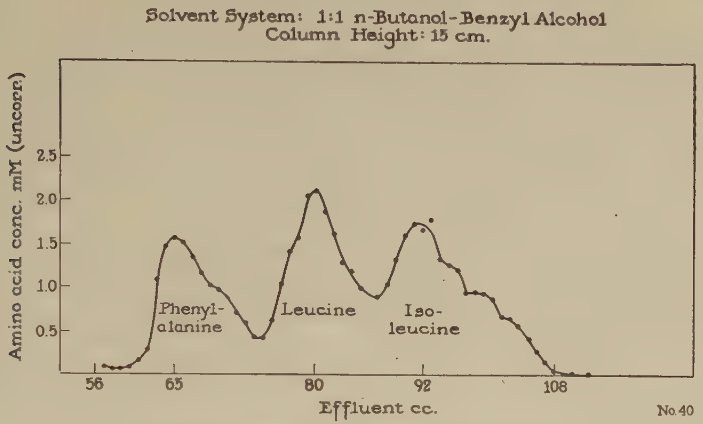

FIGURE 3. Results on an unsatisfactory column.

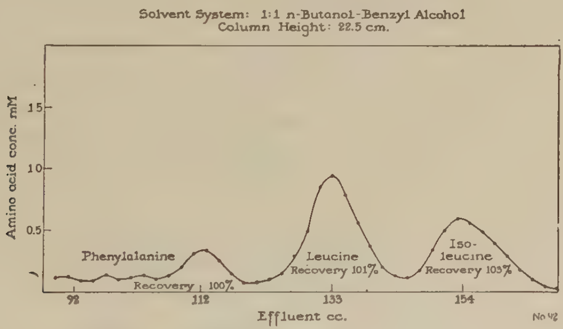

FIGURE 4. Anomalous behavior of the first band.

A difficulty which bothered us for some time is illustrated in FIGURE 4. The *first* band, phenylalanine in this case, exhibits an anomalous behavior. There is a long segment of the band running 20 cc. ahead of the peak. The second and third peaks are symmetrical. If an aliphatic amine or 8-hydroxyquinoline is allowed to run through the column ahead of the first amino acid band, the effect can be eliminated. Both of these substances are capable of forming complexes with metals. The use of

8-hydroxyquinoline has proved to be essential for the procurement of satisfactory results with all of the amino acid bands, when samples of starch are used containing significantly higher traces of inorganic impurities than the starch used in this experiment.

It is possible that the anomaly is similar to that noted by Consden, Gordon, and Martin with the paper-strip chromatogram, and ascribed to the presence of traces of copper.[4] Cupron, which abolished the effect with paper, is, however, ineffective on the starch column.

The behavior of the faster-moving amino acids in three solvent systems is given in FIGURE 5. The solvents are butanol-water, butanol-0.5N HCl, and 1:1 butanol:benzyl alcohol-water. In the first curve, which represents an early experiment with butanol-water, the shoulder on the leucine peak is the result of the effect of metals mentioned above. In the second curve, illustrating an experiment in which butanol-0.5N HCl is the solvent system, the sequence of the amino acid bands is altered. In this instance, the amino acid hydrochlorides are fractionated. In reference to the third

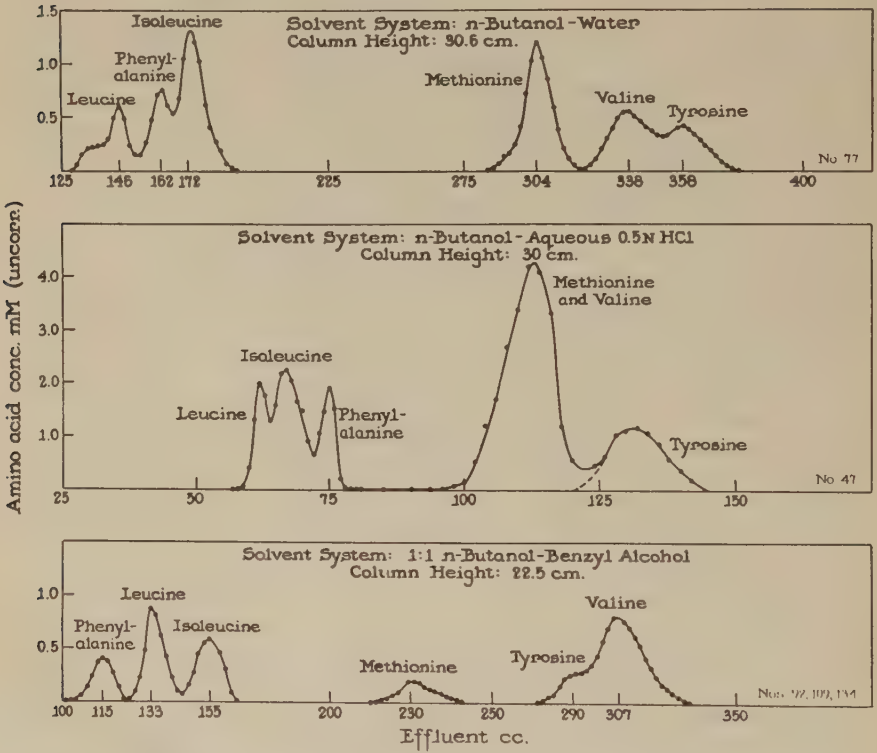

FIGURE 5. Separation of phenylalanine, leucine, isoleucine, methionine, valine, and tyrosine.

curve, as found by Consden, Gordon, and Martin on paper strips, the use of 1:1 butanol:benzyl alcohol serves to increase the band rates of the aromatic amino acids relative to the rates of the aliphatic ones. Thus, phenylalanine moves ahead of leucine and isoleucine, and tyrosine emerges ahead of valine. A more complete resolution of these three amino acids is attained in the butanol:benzyl alcohol system than in other solvent mixtures which have been studied. The recoveries of methionine, however, run low in butanol:benzyl alcohol experiments. Oxidation of the methionine sulfur by traces of impurities in the benzyl alcohol is believed to be responsible for the low yields. Satisfactory recoveries are obtained with methionine in plain butanol. It should be mentioned that tryptophane, if present, would emerge near the phenylalanine band in all three solvents, and would show up in the valley between phenylalanine and leucine in butanol:benzyl alcohol. These amino acids can be separated from one another by re-chromatographing the mixture on a separate column, as will be mentioned later. In acid hydrolysates of proteins, the problem does not arise, since tryptophane is decomposed during the hydrolytic process.

Before discussing the amino acids which appear later in the amino acid "spectrum," some of the fundamental theoretical points raised by the results shown in FIGURE 5 merit consideration. In its original formulation, "partition chromatography" was conceived to be a technique which permitted counter-current liquid-liquid extraction to be carried out as a simple column filtration with one of the phases immobilized on an *inert* support. The data in TABLE 1 serve to indicate the extent to which this conception of the operation of the column agrees with the experimental facts. In this table, the distribution coefficients of several amino acids are compared with their rates of movement on starch columns. In the first section of the table, the distribution coefficients of the amino acids in butanol-water as determined in the separatory funnel are listed in decreasing order.[8] The distribution coefficients from the rates of movement of the amino acids on the column have been calculated in each case by the method of Martin and Synge.[10] The equations employed in these calculations were derived on the assumption that the amino acids are being separated by a liquid-liquid distribution process. For leucine, isoleucine, and valine the distribution coefficients calculated from the column are somewhat higher than those found in the separatory funnel, but the relative distribution coefficients are similar in the two cases. These data support those reported by Synge,[3] who found that the distribution coefficients of leucine, valine, and alanine, calculated from starch columns, agreed fairly well with the values determined by England and Cohn[11] by the separatory funnel technique. For the simple aliphatic monoamino acids, therefore, with butanol as the solvent, the agreement between experiment and theory would make it appear that starch is serving as a relatively inert support for the aqueous phase.

TABLE 1

COMPARISON OF DISTRIBUTION COEFFICIENTS OF SEVERAL AMINO ACIDS WITH THEIR RATES OF MOVEMENT ON STARCH COLUMNS

Solvent System: *n*-Butanol-Water

| Amino Acid | Tryptophane | Phenylalanine | Leucine | Isoleucine | Tyrosine | Methionine | Valine |
|---|---|---|---|---|---|---|---|
| *Distribution coefficient in separatory funnel* * | 0.56 | 0.240 | 0.181 | 0.132 | 0.117 | 0.091 | 0.056 |
| *Distribution coefficient calculated from the column* † | ca. 0.22 | 0.20 | 0.22 | 0.18 | 0.080 | 0.096 | 0.086 |
| *Order of emergence from the column* | 2 | 3 | 1 | 4 | 7 | 5 | 6 |
| *Order of band rates on paper* ‡ | 4 | 1 | 2 | 3 | 5 | 6 | 7 |

Solvent System: 1:1 *n*-Butanol: Benzyl Alcohol-Water

| Amino Acid | Tryptophane | Phenylalanine | Leucine | Tyrosine | Isoleucine | Methionine | Valine |
|---|---|---|---|---|---|---|---|
| *Distribution coefficient in separatory funnel* * | 0.60 | 0.270 | 0.123 | 0.104 | 0.097 | 0.082 | 0.044 |
| *Distribution coefficient calculated from the column* † | 0.20 | 0.21 | 0.18 | 0.072 | 0.15 | 0.094 | 0.068 |
| *Order of emergence from the column* | 2 | 1 | 3 | 6 | 4 | 5 | 7 |
| *Order of band rates on paper* ‡ | 3 | 1 | 2 | 6 | 4 | 5 | 7 |

* Distribution coefficient $= \frac{\text{conc. in non-aqueous phase}}{\text{conc. in aqueous phase}}$, determined with 0.01 M amino acid solutions at 25°C. Accuracy ca. ± 2%[8].

† Calculated by the method of Martin & Synge[10].

‡ From data of Consden, Gordon, & Martin[4].

However, when the distribution coefficients in butanol-water of all of the amino acids given in TABLE 1 are determined, a number of deviations from the theory become apparent. If the starch column were operating as a true liquid-liquid distribution system, the amino acids should, in each case, emerge in the order of the distribution coefficients as listed in decreasing order. The actual orders of emergence are those shown in FIGURE 5 and listed in the table. The scrambling of the order results from the behavior of phenylalanine, tryptophane, and tyrosine, which do not travel on the column at the rate which would be predicted from the distribution coefficients. Tryptophane should be as far ahead of phenylalanine as isoleucine is ahead of valine; actually, it is behind leucine. Phenylalanine should be well ahead of leucine, instead of behind it, and tyrosine should be between isoleucine and methionine, whereas, actually, it emerges last. Furthermore, the effect upon the aromatic amino acids is not peculiar to starch. As may be seen from the data of Consden, Gordon, and Martin, listed in TABLE 1, similar anomalies are noted with cellulose in the paper-strip chromatogram. Tryptophane, for example, is markedly out of order in this instance.

The data for 1:1 butanol:benzyl alcohol are given in the second section of TABLE 1. In the case of the paper-strip chromatogram, the deviations from the expected order are more marked than is the case in butanol. The same type of deviation from theory has also been demonstrated for the butanol-0.5N HCl system, for which the orders of emergence are given in FIGURE 5.

The conclusion to be derived from these data is unequivocal, namely, that starch does not serve merely as an inert support for the aqueous phase.

In this connection, the following simple experiments have been carried out in which no organic solvent whatever has been employed. An aqueous solution of glycine and alanine has been passed through a column composed of only starch and water (FIGURE 6). The separation is quantitative. The experiment has been repeated with leucine, phenylalanine, and tryptophane (FIGURE 7). Again, with no organic solvent present, complete separation is obtained. That this effect is not pH-sensitive, is demonstrated by the dotted curve in the figure. This second curve was obtained by fractionating the hydrochlorides of the amino acids on a column made up with starch and aqueous 0.1N hydrochloric acid.

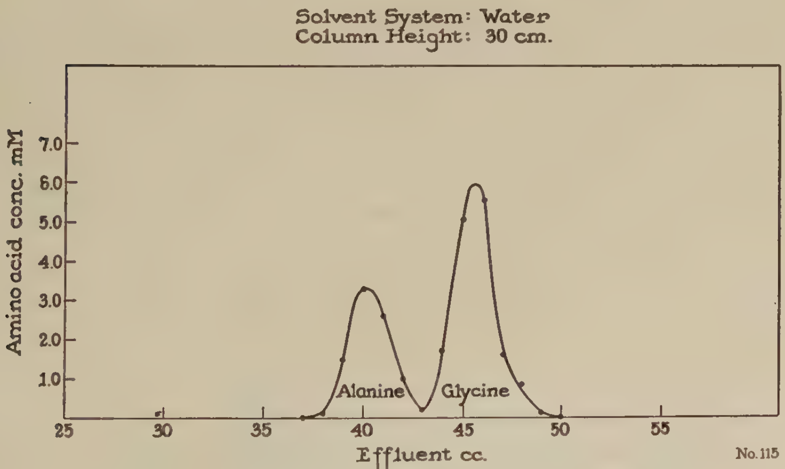

FIGURE 6. Separation of glycine and alanine on a water-starch column.

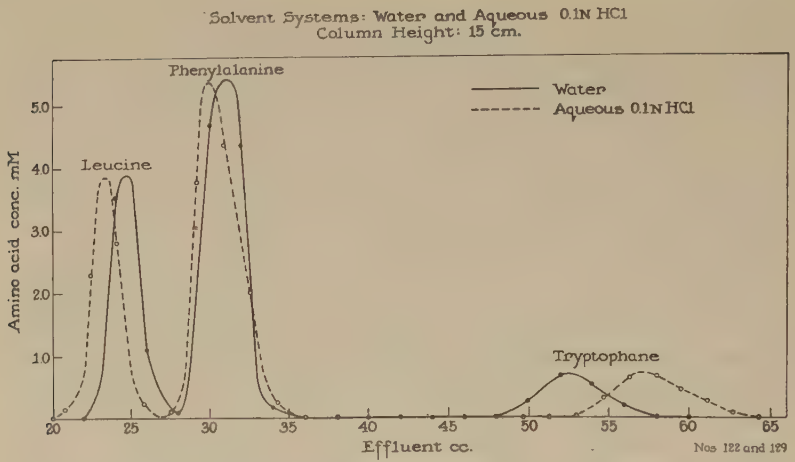

FIGURE 7. Separation of leucine, phenylalanine, and tryptophane.

In this instance, the fractionation can be a useful one for separating tryptophane from phenylalanine and leucine, which, as was mentioned earlier, is a separation difficult to accomplish with organic solvents. Thus, some of the experiments which were run primarily for their theoretical interest led to the realization that useful results with amino acids may be obtained on starch columns using water-miscible solvents. This point will come up again in the discussion of the chromatography of the basic amino acids. There are indications from the work of Consden, Gordon, and Martin that amino acids can also be fractionated on paper strips with the aid of solvents miscible with water.[4]

From the foregoing, it would appear that the starch column possesses properties characteristic of both an adsorption chromatogram and a partition chromatogram. In view of the known adsorptive properties of the starch granule,[12] this is not an unexpected finding. The data indicate clearly that further work is necessary in order to elucidate the role of adsorption in the performance of both the starch and the cellulose chromatograms.

Two further points which bear upon the theoretical interpretation warrant mention. The first point concerns the symmetry of the effluent concentration curves. The symmetry is indicative of a fairly linear isotherm, irrespective of the definition of the process by which the fractionation is attained. The second point of importance is the reproducibility of the absolute position in the "spectrum" of a given amino acid band. When butanol-benzyl alcohol is employed as the solvent, the amino acid valine, for example, in the presence of twenty other components, emerges at the same position as it does when it is the only amino acid on the column. The same holds true for all the other amino acids which have been tested thus far. This situation is in contrast to that frequently encountered in adsorption chromatography, where the behavior of a given substance is markedly altered by the presence of other components.[13, 14]

Before leaving the discussion of the theory of chromatography on starch, it is of interest to consider the order of magnitude of the resolving power of the starch column. The case of leucine and isoleucine in 1:1 butanol:benzyl alcohol is an instance where the ratios of the two distribution coefficients calculated from the column and measured in the separatory funnel are similar. For purposes of comparison, without making any assumptions as to the mechanism of action of the column, it is possible to calculate how many "plates" would be required to achieve, by liquid-liquid distribution in a series of separatory funnels, the same degree of separation of these two amino acids attained on the column. The methods of calculation of Martin and Synge[10] and of Craig,[5] taken in conjunction with our data, point to the fact that the starch column is performing a task which would require several thousand separatory funnel "plates."

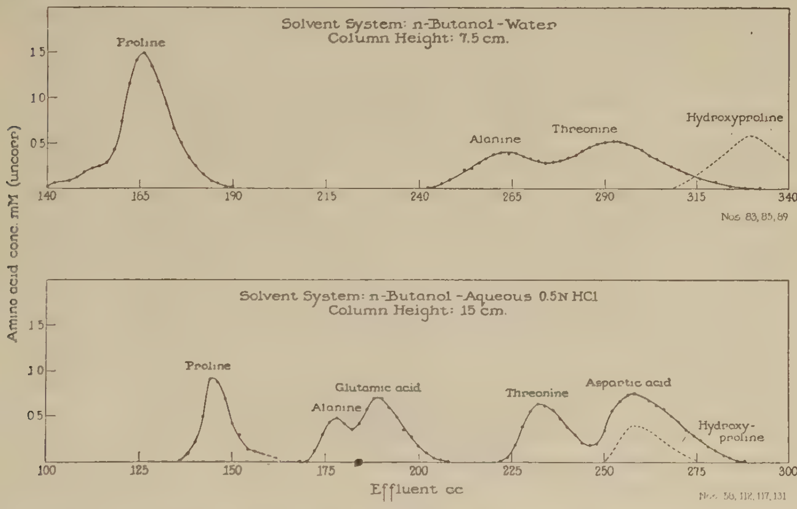

**FIGURE 8.** Separation of amino acids with band rates in the range of that of alanine.

The comparison of the performance of the column with that of a several thousand "plate" system is a realistic one for revealing, qualitatively, the extremely high resolving power inherent in the starch chromatogram and for indicating the position of this technique among the tools available to the organic chemist for the study of complex multicomponent systems.

In returning to the discussion of the amino acid "spectrum," it should be stated that there has been no opportunity, as yet, to study the slower-moving bands as thoroughly as the phenylalanine-through-valine group has been examined. The results which follow are of a more preliminary nature and are presented to show that it has proved possible to cover the whole spectrum, although the degree of resolution is not satisfactory in all instances. Two curves illustrating the behavior of a number of the amino acids falling in the next group to emerge from the column are given in FIGURE 8. A shorter column is used, and the incompletely separated faster bands, if present, are accumulated in a fore-fraction. In butanol-water, proline is the next amino acid to emerge after tyrosine and valine, and it is well separated from alanine, threonine, and hydroxyproline. A different picture is obtained employing the acid solvent mixture, butanol-0.5N HCl. Alanine is pulled well apart from threonine. Hydroxyproline has about the same rate of travel as aspartic acid. Most of these overlaps are resolvable by re-chromatographing the zones in question. It is probable that further study of solvent systems will lead to results which will reduce the number of instances where this would be necessary.

Serine and glycine are the most slowly-moving members of the mono-amino monocarboxylic acid series. To speed up their rate of movement, *n*-propanol can be used in preference to *n*-butanol (FIGURE 9). In neither of these solvents, however, has complete resolution been obtained.

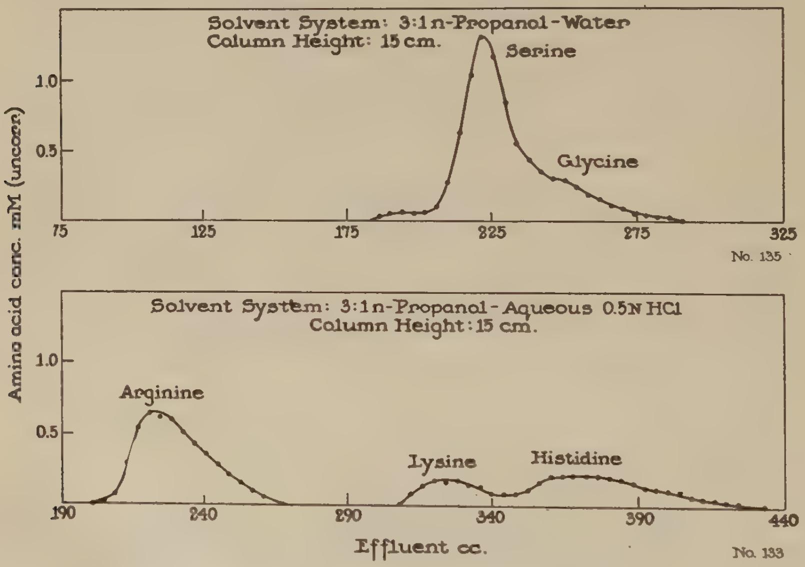

FIGURE 9. Separation of serine, glycine, and the basic amino acids.

The basic amino acids present a special problem. In butanol-water and in butanol-0.5N HCl, arginine, lysine, and histidine do not move at an appreciable rate. Even in plain water, arginine and lysine are bound strongly near the top of the column. The addition of HCl to the water causes all three basic amino acids to move rapidly. The combination of aqueous HCl and propanol provides a solvent mixture which gives a satisfactory rate of travel for the basic amino acids and yields reasonably complete separation (FIGURE 9). This preliminary experiment is a further example of the use of water-miscible instead of water-immiscible solvents in the starch chromatogram.

Relatively few experiments have been carried out, to date, on the behavior of peptides on the column. One example can be given of the separation of the isomeric dipeptides leucylglycine and glycylleucine (FIGURE 10).

The results of an experiment on a hydrolysate of β-lactoglobulin are given in FIGURE 11, as an example of one of the problems to which this technique can be applied. The first six amino acids in the spectrum appear in the usual order. In this mixture of more than twenty components, the positions of the bands are the same as when the amino acids

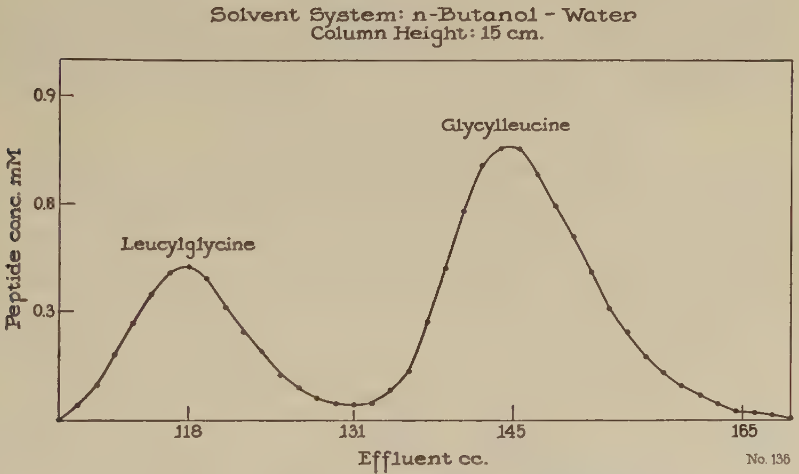

FIGURE 10. Separation of leucylglycine and glycylleucine.

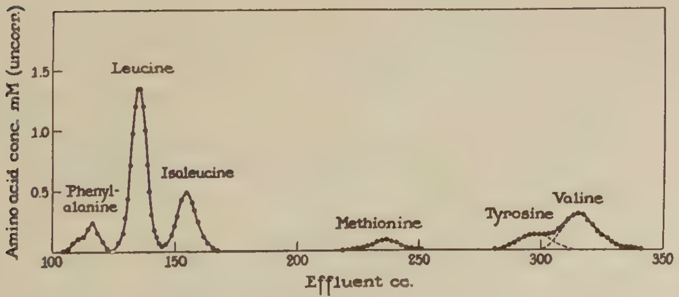

FIGURE 11. Separation of amino acids in a hydrolysate of β-lactoglobulin.

are run individually. Integration of the curves gives the following values for the amino acid composition of $\beta$-lactoglobulin (gm. per 100 gm. of protein)[8]: phenylalanine, 3.78; leucine, 15.5; isoleucine, 5.86; tyrosine, 3.64; and valine, 5.62. The values are in good agreement with the results reported by Brand *et al.*,[15] with the exception that the starch chromatogram indicates a lower value for the isoleucine content. The results which have been obtained with this method of fractionation support the conclusion that chromatography of free amino acids on starch columns, as introduced by the work of Martin and Synge and Elsden, and as extended in the experiments outlined above, is capable of yielding sound quantitative data as well as qualitative pictures of the composition of mixtures of amino acids and related compounds. The studies are still in their relatively early stages, and much is yet to be learned concerning both the potentialities and the limitations of the method in its application to biochemical problems.

In conclusion, the authors wish to acknowledge the valuable assistance of Miss Enid Mellquist and Mr. H. R. Richter in the course of this work, and the microanalytical aid of Dr. Adalbert Elek of the Institute.

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# A REVIEW OF FRACTIONATION OF MIXTURES BY FOAM FORMATION

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Chromatographic methods employing solid adsorbents are now frequently used, but the possibilities of fractionations and purifications with foams (at air-liquid interfaces) and emulsions (at liquid-liquid interfaces) have hardly been investigated.

It has been known for some time that the components of certain solutions are adsorbed by the foams which these solutions can form, as well as at liquid-liquid interfaces. Gibbs' Adsorption Equation* indicates increased concentration of a single surface active material at the interface by reversible adsorption. In a number of cases, the measured adsorption is equal to or many times the calculated amount.

## MEASUREMENT OF ADSORPTION AT LIQUID-LIQUID AND GAS-LIQUID INTERFACES

W. C. M. Lewis[1] estimated the adsorption of sodium glycocholate, sodium oleate, congo red, and methyl orange for aqueous solutions against hydrocarbon oil and found it to be about sixty times the calculated value. The adsorption was about $5 \times 10^{-6}$ gm. per cm.$^2$

Donnan and Barker[2] found the adsorption at gas-liquid interfaces, as indicated by surface concentration, to be of the order of $10^{-7}$ gm. per cm.$^2$ for dilute solutions (0.002 to 0.008 per cent) of nonylic acid. These values agree in order of magnitude with those calculated from Gibbs' Equation.

McBain and DuBois[3] measured adsorption at the surface of aqueous solutions for isoamyl alcohol, acetic, butyric, caproic and nonylic acids, phenol, *p*-toluidine, resorcinol, thymol, and camphor. They found that gas bubbles passing through a solution carry from two to eight times as much solute as is predicted by Gibbs' Equation.

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* $\Gamma = -\frac{a}{RT} \times \frac{d\gamma}{da}$, where $\Gamma$ is excess solute per unit surface, $a$ = activity of solute, and $\frac{d\gamma}{da}$ = rate of change of surface tension with activity. Where activity coefficients do not change with concentration, concentrations are used instead of activities.

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## PURIFICATION OF MIXTURES BY SELECTIVE ADSORPTION IN FOAM OR EMULSION

The above values show the extent of adsorption for a number of substances. For mixtures, fractionation of the constituents can be obtained by selective adsorption at air-liquid interfaces (foam) or liquid-liquid interfaces (emulsions). This suggests the use of foams for the removal of positively adsorbed substances from solution. However, very little quantitative work along these lines has been reported, and for adsorption in emulsions even less data are available.

In the following discussion, the recent papers[4-10] which indicate some of the factors involved in such separations will be considered.

Miles and Shedlovsky[4, 5, 6] have shown the effects of small amounts of certain impurities which lead to minima in surface tension-concentration curves. Such minima can be attributed to the presence of at least two surface active constituents in the same solution. Selective adsorption at air-liquid interfaces (foam) or benzene-liquid interfaces (emulsion) have indicated these minima to occur at bulk concentrations where the relative surface concentrations of the component present in smaller amount was at a maximum.

As an example of selective adsorption at air-liquid interfaces, the data given by Miles[5] may be considered. Known volumes of foam of uniform bubble size were produced by passing clean air, at constant pressure, through a single glass orifice into 50 ml. of solution. The interfacial area was approximately 2 x $10^4$ cm.$^2$ for a full cylinder of foam, as calculated from the bubble volume, which was estimated as 0.017 ml., and the cylinder volume of 1100 ml. After forming the foam, the solutions were brought to what was believed to be a steady state with respect to the newly created surface, by agitating the bulk liquid gently in contact with the interface for several minutes.

Curve 1 of FIGURE 1 represents the surface tension-concentration relationships for pure sodium lauryl sulfate which had been ether-extracted in the dry state for 36 hours in a Soxhlet apparatus. Curve 2 illustrates the minimum obtained when pure sodium lauryl sulfate is contaminated with 0.5 per cent dodecanol, on the total solids basis. Point A′ is the value obtained after forming a full cylinder of foam from 50 ml. of 0.015 molal solution (surface tension A) and then diluting to 0.0075 molal. Point B′ is the value obtained when the same procedure is followed starting with 0.010 molal, (surface tension B) and then diluting to 0.0075 molal after selective adsorption. Point C′ was obtained by the same steps as Points A′ and B′, except that the starting and final concentrations were

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[4] **Miles, G. D. & L. Shedlovsky.** J. Phys. Chem. **48:** 57. 1944.

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[9] **Schütz, F.** Trans. Far. Soc. **42:** 437–444. 1946. French Patent 835,439. Dec. 21, 1938.

[10] **Bader, R. & F. Schütz.** Trans. Far. Soc. **42:** 571–76. 1946.

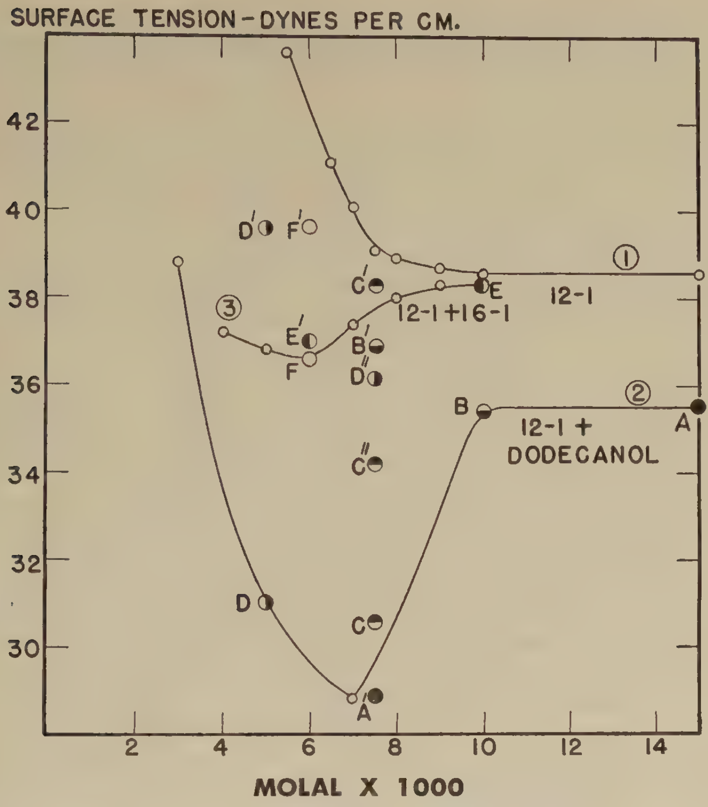

FIGURE 1. Effect of foam extraction on surface tension of sodium dodecyl sulfate (12-1) + 0.5% dodecanol and sodium dodecyl sulfate (12-1) + 1% sodium hexadecyl sulfate (16-1). (Miles [5]).

0.0075 molal. Point C″ was obtained in the same way as C′, except that one-third the volume of foam was employed for C″ as compared to C′. Fifty ml. of 0.005 molal solution, surface tension (D), was used to make a full cylinder of foam and the surface tension (D′) of the bulk liquid measured. On increasing the concentration to 0.0075 molal by the addition of pure sodium lauryl sulfate to this solution, a surface tension D″ was obtained. Point F′ was obtained by the same adsorption procedure, starting from a 0.006 molal solution with surface tension F.

The examples given are typical of other mixtures, and surface tension-concentration and interfacial tension-concentration curves are effective guides to the concentrations most suitable for purification by selective adsorption, as well as to show the presence of even minute amounts of certain surface active constituents. This gives criteria of purity not obtainable from the measurement of bulk properties. By studying both the surface tension-concentration and interfacial tension-concentration curves, using a non-aqueous liquid for the latter, it is sometimes possible to choose the method of purification to be used. If the surface tension-concentration curve shows a minimum, selective adsorption in foam is indicated, whereas, if the interfacial tension-concentration curve shows a minimum, selective adsorption in an emulsion should be tried. In either case, the most effective removal of the impurities is obtained at the concentration corresponding to the minimum. For example, we have found that addition of dodecanol to a solution of sodium dodecanol sulfate shows a pronounced minimum in the surface tension-concentration curve, but no minimum in the interfacial tension-concentration curve (solution against benzene). The dodecanol can be completely removed from this solution by the use of foam, whereas a benzene emulsion is not suitable for this purpose.

The procedure for separations with foams or emulsions will depend on whether it is desired to recover the material adsorbed in the foam or that remaining in the solution. For removal, from the solution, of materials which are selectively adsorbed in the foam, the method which we use at present consists of preparing a column of foam in a cylindrical pyrex jacketed tube 46 cm. long and 3.7 cm. in diameter, from another jacketed tube placed below the first one. The lower tube is 17 cm. long and 2.8 cm. in the widest portion. Filtered air is bubbled through a stainless steel perforated cup "spinnerette" (40 holes, 0.0032 inches in diameter). Surface tension measurements are made of the solution at various concentrations after it has been poured through the foam, and about 15 minutes are allowed for drainage. In some cases, repeating the operation of pouring the solution through the foam gives improved selective adsorption and a more complete approach to a steady state.

For selective adsorption at benzene-liquid interfaces, mentioned previously,[5] relatively small portions of the solution were gently agitated with the emulsion, whereupon samples of the bulk solution were withdrawn and their interfacial tensions compared with the values obtained for untreated solutions.

These selective adsorption experiments were made with mixtures where one constituent was present in small amounts. It is not known whether a similar procedure could be used satisfactorily for binary mixtures where both components were present in relatively large amounts. Furthermore, its applicability to multicomponent and more complex mixtures has not been investigated.

## ABRIBAT'S METHODS FOR FRACTIONATION OF MIXTURES IN FOAM

M. Abribat[7] describes certain methods of fractionation of various materials using foam. He emphasizes that drying of foam by drainage is responsible for the enrichment of foams with respect to certain constituents in the solution, and he insists that the separation of numerous constituents is primarily a function of the difference in the flow characteristics at the gas-liquid interfaces and of the bulk liquid. Furthermore, he claims that this separation does not necessarily correspond to the surface activity of the constituents involved.

**Procedure.** The foam is made by bubbling air or nitrogen through a porous cup (*E*) at the bottom of a vertical glass tube (FIGURE 2). The gas bubbles should be as small as possible, so that they do not rise too rapidly, allowing adequate time for adsorption.

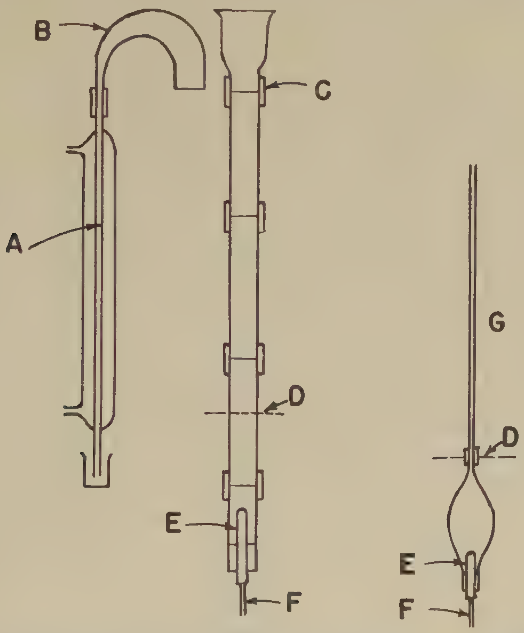

FIGURE 2. Foam fractionation apparatus, after Abribat[7]. *A*, defoaming tube; *B*, connecting tube; *C*, rubber sleeve; *D*, liquid level; *E*, porous cup; *F*, gas inlet; *G*, thin wall apparatus for solutions which form small amount of foam.

Since certain constituents of the original solution are concentrated in the foam, the amount of foam which can be formed from the solution decreases as it progressively loses those constituents which cause it to foam. In order to obtain effective separations, it is important to allow the foam to drain as much as possible. For some foams whose films have a high surface viscosity, such as protein foams, considerable liquid remains even after drainage has been essentially complete.

The height of the column of foam, *H,* from the original liquid level and the drop in the liquid level, *h,* are recorded as a function of time, with the gas bubbling at a fixed pressure (FIGURE 3). In general, *H* passes through a maximum for a constant gas pressure. These measurements are used by Abribat as a guide to the procedure to be followed in foam fractionation. He considers the type of data shown in FIGURE 3 as indicating

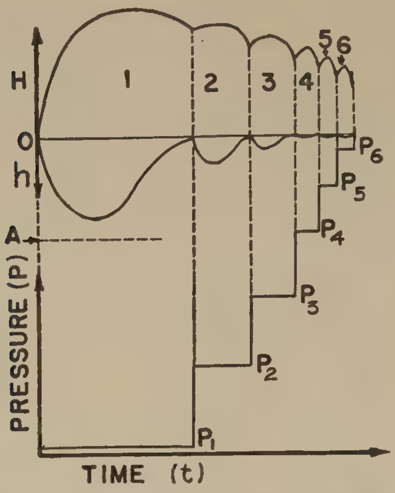

FIGURE 3. Schematic diagram for operation of foam fractionation, after Abribat[7]. *H*, height of column of foam; *h*, drop in liquid level.

relative drainage rates, but it seems that both the foam volume and the maximum total amount of liquid held in the foam, which is proportional to $h$, decrease for each increase in pressure at which the gas is bubbled.

To carry out an experiment, the bubbling of gas is first started at low pressures which are progressively increased until $H$ is constant. The pressure should always be below the limit which corresponds to the appearance of eddy currents of bubbles in the foam. The porous cup should be at least 5 or 6 cm. below the liquid level for a tube of 5 cm. inside diameter.

After a few preliminary trials, a suitable pressure, $P_1$ is chosen (FIGURE 3). Then, $t_1$ is the time required to reach a steady state when foam breakdown is compensated by foam formation and $H$ is constant. The pressure is increased to $P_2$ and the measurements of $H$ and $h$ against $t$ are repeated.

The values of $t$ become progressively shorter. After depletion of the solution of surface active constituents, and without diminishing the gas pressure, the sections of foam are removed. This can be done in a single operation if the apparatus is large enough. Otherwise, the sections of foam may be removed in steps corresponding to $t_1$, $t_2$, $t_3$, etc. For this purpose, the connecting tube (FIGURE 2, *B*) is placed over any one of the sections and the required foam fraction is collected in the defoaming tube.

Abribat's apparatus (FIGURE 2) is made with long cylindrical sections connected with ground joints or rubber sleeves, and the dimensions range from narrow columns several feet high to wide ones reaching a total of perhaps 20 feet. The size suitable for all operations cannot be specified, because this varies with the amount of foam formed. A relatively narrow tube is recommended for solutions which form only a small amount of foam, such as dilute solutions of proteins, virus, and serums. If a vertical tube with a sufficiently thin wall is used (FIGURE 2, *G*), the column may be cut into appropriate sections with a glass cutter at the end of the experiment. If necessary, the fractions obtained can again be treated separately by forming new foams, either after dilution or directly in a smaller apparatus. Other details of the apparatus are indicated in FIGURE 2. A jacketed apparatus is used to carry out experiments at various temperatures.

**Effect of Concentration of Solutions and of Bulk and Surface Viscosity.** According to Abribat, in many cases and especially for protein solutions, the fractionation by foam drainage is more complete for dilute solutions, as long as they still form rigid surface films. In this case, the difference between surface and bulk viscosity is greater than for more concentrated solutions. The addition of materials with high bulk viscosity is usually undesirable, since it merely interferes with drainage.

If mutual adsorption does not occur, one may obtain selective separation in foam of a particular material by altering the temperature. This effect is apparently due to an abrupt decrease in surface viscosity.

**Eddy Currents in Foams During Fractionation.** If eddy currents of bubbles are formed in the foam in Abribat's apparatus, even for a short time, this may be sufficient reason to ruin a separation previously obtained. Such disturbances are greater in the more fluid portions of the foam, which have relatively large amounts of liquid, and are more easily produced for larger cross-sections of the foam column and for higher rates of gas flow. Abribat avoids eddy currents by using a device[8] which allows for a convenient agitation of the column of foam. The rotation of a series of vertical coaxial stirrers tends to carry each bubble in a circular path in a horizontal plane, and, as the foam rises, the path becomes helical. This arrangement is claimed to have the further advantage of increasing the length of travel of individual foam bubbles, besides which it allows drainage of the foam in a shorter column than would be necessary in the absence of such stirring. With this arrangement, the various fractions are sharply separated in the column.

The eddy currents are due to the various-sized bubbles formed with the porous cup. Large bubbles travel faster than small ones and collect at the top of the foam column. We have found that foams of uniform bubble size are formed by using a single orifice, or "spinnerettes." Such foams should be a distinct advantage in foam fractionations, since difficulties due to eddy currents would be largely eliminated.

**Applications:**

PROTEINS. For proteins, the rigidity of the interfacial films of the foams is a function of both pH and temperature. Other factors being equal, the most suitable conditions for foam fractionation obtain at the isoelectric pH. However, the temperature should not exceed a certain value above which some proteins such as gelatin do not form viscous films and the foam persistence is low. Furthermore, foam enrichment by foam drainage is almost nil. In such cases, above a certain temperature, separation is not improved at any concentration.

Gelatin has been separated by Abribat from heat-denaturable proteins, such as albumins and globulins. These proteins constitute the products obtained at the beginning of the fractionation, and some of them flocculate irreversibly in the foams. Gelatin is not denatured by heating its solution, nor is it denatured by foam formation.

COLLARGOL. For a sol of collargol, during the foam formation, the liquid is rapidly decreased in concentration of colloidal silver. No flocculation is produced in the liquid during the operation and until the removal of the last traces of silver. If proteins, which can be denatured by foaming, are present, the constituents of the foam may flocculate irreversibly.

METHYL VIOLET DYES. Abribat repeated the experiments of Kenrick[11] with a mixture of methyl violet (tri-, tetra-, penta- and hexamethyl-pararosanilines). The top fraction of the foam was almost entirely composed of the hexamethyl derivative (crystal violet) which had a characteristic violet color, whereas the product at the lower section of the foam was red-purple.

Towards the end of the operation, the fractionation could be accelerated and considerably improved by adding a very small amount of gelatin, which increased the volume of foam. Beyond mentioning the difference in color, Abribat does not indicate the purity of the fractions obtained.

MIXTURE OF SAPONIN, GELATIN, AND SODIUM OLEATE. When a dilute solution of gelatin containing sodium oleate was subjected to a foam-forming treatment, the top fractions of the foam column were composed exclusively of gelatin. The oleate was separated, subsequently, from an alkaline solution to avoid hydrolysis of the sodium oleate. In order to obtain suitable properties of the gelatin films in the foam, it was necessary to add about ½ cc. per liter of ethanol to the solution.

During the foaming of a solution of gelatin and saponin, the saponin was found in the top fraction. If the solution also contained sodium oleate, the dissolved substances were at least partly concentrated in the foam column in the following order: saponin, gelatin, sodium oleate. This appears to be in accordance with the rigidities of the interfacial films formed. According to Abribat, this does not follow the individual surface activities of the different substances, when these are taken separately.

OTHER MATERIALS. Abribat separated fatty acids closely related to each other in constitution and number of carbon atoms. He suggests the use of the method for various protein and lipoid substances, blood serum, various plasma, as well as for the separation of enzymes, virus, or hormones.

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## SCHÜTZ'S METHODS FOR FRACTIONATION OF MIXTURES IN FOAM

**Procedure.** F. Schütz[9, 10] and co-workers report on the fractionation by adsorption and crystallization on foam. Their method consists in bubbling nitrogen through a single orifice at the bottom of a relatively short column, 28 cms. high and 3.5 cms. in diameter (FIGURE 4), and the foam is allowed to go over a bend at the top of the tube into a side vessel. The height of the liquid through which each bubble rises, and the time the bubble rests in contact with the liquid, have to be sufficient for surface equilibrium to be approached. Schütz considers this condition satisfied with the liquid level about half-way up, but he does not give the basis for his statement.

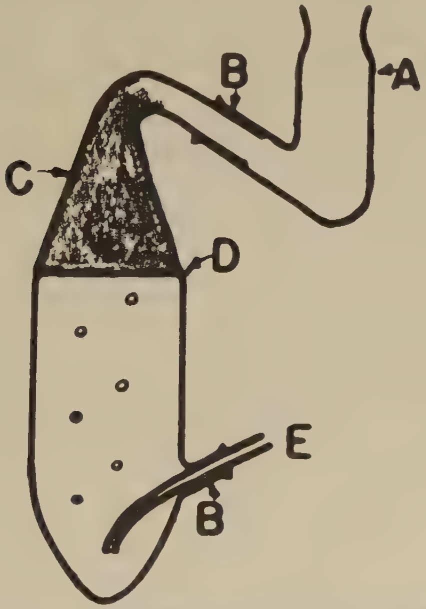

FIGURE 4. Foam fractionation apparatus, after Schütz[9].

The rate of gas flow is adjusted so that the bubbles reach the upper overflow bend in the tube. Schütz claims that this has the advantage of foam automatically ceasing to be carried over if its stability suddenly decreases or increases, when the surface active material collected from the foam has all been removed from the solution. At this point, the receiver is changed, the gas pressure is increased, and the next fraction collected. This accounts only for the volume of foam produced, regardless of bubble sizes and other properties, such as drainage characteristics.

If stable foam is not obtained from a solution, stability can sometimes be induced by adding a liquid which reduces the solubility of the substance to be adsorbed. Schütz finds that aqueous solutions of inulin hardly form foams, but they give very stable foams if prepared with 1.65 per cent ethanol or 10 per cent dioxane.

He notes that, unless there are indications from "peaks" of "foam time"* curves,[12] it is best to make the foam at as low a concentration as possible. This corresponds with the observations made by Abribat with regard to concentration.

In nearly all cases, some solid matter is found in the foam fraction, and this often leads to a more efficient fractionation. However, in foaming proteins, much of the solid matter in the foam fractions is surface-denatured protein. The foam can be broken by centrifuging or by drying in a desiccator.

Schütz recommends the choice of "peaks" of "foam time" against concentration curves[12] as a guide to the concentrations to be used for foaming. For the purification of proteins and bile salts, better fractionation was obtained at the isoelectric pH.

The surface tension of the foam fractions was lower than that of the original solution, and efficient fractionation was shown by an increase in the surface tension of the solution. However, Schütz considers that "foam time" is a more sensitive indicator than surface tension of when to change the receiver. On the other hand, we have seen before how in our work[4, 5, 6] the minima in surface tension concentration curves correspond to optimum selective fractionation of certain components from which the foam was formed.

**Effect of Temperature.** Thermolabile substances like enzyme solutions can be converted into foam at 0°C. For this purpose, the incoming gas should be pre-cooled. Changes in both bulk and surface properties may change considerably with temperature, as indicated, for example, in the drainage characteristics of foams.

The rates of flow of liquid through foams decrease with increase in both surface and bulk viscosities,[13] both of which are affected by changes in temperature.

If an increase in temperature decreases the viscosity without causing other pronounced changes in properties, it is advisable to use higher temperatures. However, Schütz finds that for some materials there is an optimum temperature, different for various materials, beyond which, at higher temperatures, fractionation by foam is no longer possible. For sodium cholate, this temperature is 36°C., and for saponin, 70°C. In this connection, it may be of interest to note that differences in drainage rates of foams for various materials become insignificant above certain temperatures, due to an abrupt decrease in surface viscosity.[13]

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* The first method given by Schütz consisted in bubbling gas through the solution by means of a capillary tube. The period between the stoppage of gas flow and the time when half the foam-covered surface became free of bubbles was called the "foam time."

For aliphatic alcohols, Schütz says that a standardized shaking method was used. After shaking was stopped, the time taken until the center of the liquid became free from bubbles was called the "foam time."

[12] **Schütz, F.** Trans. Far Soc. **38:** 85. 1942; **38:** 94. 1942.

[13] **Miles, G. D., L. Shedlovsky, & J. Ross.** J. Phys. Chem. **49:** 93-107. 1945.

**Size of Bubbles and Rate of Drainage.** The size of the bubbles can be varied by using jets with different openings. As a rule, smaller bubbles are more advantageous since they correspond to more surface for the same total gas volume. However, Schütz found that, for saponin, smaller bubbles remove less material than larger ones. This was probably due to the much slower drainage rate for the smaller-size bubbles.[13]

**Rate of Foam Formation.** Since Schütz used "foam time"[12] as a guide in the technique and this depends on the bubble size, sintered glass was found unsuitable, as it produces various bubble sizes which mask the observed changes in foam stability, quite apart from the fact that slow foaming cannot easily be achieved by means of such a filter. Somewhat better results can be obtained by using a multijet, with 12-20 openings of approximately equal diameter. In using more than one opening, the size of the apparatus must be increased, for, otherwise, the greater amount of foam produced per unit time reduces the desired preferential adsorption of one constituent. In our work,[13] stainless steel "spinnerettes" of the type used in making rayon (for example, 40 holes, $320 \times 10^{-5}$ inches in diameter $\pm 5 \times 10^{-5}$ inches) are used for obtaining foams of uniform bubble size.

By forming foam from a solution of sodium tauro-glyco-cholate by a single jet, crystallization of fatty acids and bile acids in the foam fractions was obtained by Schütz, but, if a multijet was used for the same solution in this apparatus, none of the above results could be obtained. This was due mainly to the increased rate of flow of gas, and to the shorter time the foam remained on the surface of the liquid and in the solution vessel. The average time one bubble took to be drawn over into the side vessel was approximately 30-40 sec. If the rate of flow was increased so that only 5 seconds were allowed, there was hardly any difference in concentration between the foam fraction and the residual solution. When a multijet was used, the height but not the width of the apparatus was increased.

Schütz sometimes found that much of the foam became stagnant in the upper part of the vessel and that newly formed foam was moving through a channel of this stagnant foam. This was often found to be a disadvantage, because the stagnant foam which should have been removed with an earlier foam fraction was sometimes quite efficient in breaking the later foam bubbles when these came in contact with it. The stagnant foam was therefore removed through an opening at the top of the column (not shown in FIGURE 4), before it was carried over the side. With solutions which initially showed very low "foaming stability", it was found better to fill the vessel to a higher level, so that bubbles had a shorter distance to travel from the surface of the liquid until they reached the side vessel.

**Applications:**

BILE SALTS. R. Bader and F. Schütz[10] describe the fractionation by adsorption and crystallization on foam of bile salts. Free bile acid was adsorbed and found in crystalline form in the foam fractions. Foam was formed from 0.4 per cent sodium cholate at 24°C. with a jet 2-3 mm. in diameter.

Impure preparations yielded crystalline fatty acids in the earlier fractions and crystalline bile acids in the later foam fractions. When the fatty acids were removed by foaming and the bile acids started to separate, there was a sudden change in "foam stability". The nitrogen pressure for the first foam fraction was less than 0.5 lbs. per square inch and was adjusted until the flow of foam entering the side vessel was not quite continuous. The lower the rate of gas flow, the greater the fractionation. The concentration of the cholic acid in the foam fractions was 3 to 6 times that of the original solution. In subsequent operations, the pressure was increased or decreased for the next fraction, depending on whether the foam volume decreased or increased.

The optimal pH for these fractionations of salts of bile acids was 6.5. Intermittent addition of hydrochloric acid solution was found to be a more satisfactory method of controlling the pH than the use of buffer solutions, because the latter completely hindered the crystallization of the free acids in the foam fractions. In other cases, the salt in the buffer solutions may alter the adsorption in the foam. The fatty acids and similar materials appeared in the first three fractions.

The foam in the side vessels was mostly broken on standing 2 to 3 hours, and the remainder was broken by centrifugation.

## DOGNON'S METHODS FOR FRACTIONATION OF CHEMICAL AND BACTERIOLOGICAL MIXTURES

A. Dognon[14, 15] discusses concentration and fractionation by forming foams for both chemical and bacteriological mixtures.

**Procedure.** In the simplest case, his apparatus consisted of a cylindrical tube 25 to 30 mm. in diameter and 50 to 60 cm. high. Drainage of foam was practically complete in the top section, if the gas pressure was regulated so that the upper boundary of the foam remained stable for a sufficiently long time. Then, the various fractions could be recovered by increasing the pressure so as to collect the successive foam fractions by overflow.

**Applications:**

BLOOD SERUM. Dognon confirmed the common observation that there was no significant separation of material in the foam above certain concentrations of solution. At higher concentrations, the adsorption was

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[14] **Dognon, A.** Actualités Scientifiques et Industrielles. Hermann et C$^{ie}$. Paris. 932: 157-172. 1942; Bull. Soc. Chim. Biol. 23: 249-63. 1941; Rev. Sci. 79: 613-19. 1941.

[15] **Dognon, A., & H. Dumontet.** C. R. Soc. Biol. 135: 884. 1941.

poor, as indicated by Gibbs' Equation, and the drainage characteristics of the foam were unfavorable for effective separations. On diluting a solution, there was an increase not only in the ratio of the foam to solution concentration but also in the total concentration in the foam.

For blood serum, concentrations above 0.5 per cent cannot be treated. The adsorption of protein in the foam may give a concentration 20 or 30 times greater than in the initial serum. Considerable denaturation takes place, especially of the globulins.

Bacterial Suspensions. A separation of bacterial suspensions by foam is regarded by Dognon as a sort of microflotation. For this purpose, a cylindrical tube, shaped on top like an inverted funnel, had a two-hole rubber stopper at the bottom which contained a porous cup. Samples of liquid were periodically removed from below and examined for optical density with a photoelectric turbidimeter.

The bacteria removed from the surface of a solid medium were made into a suspension in the medium chosen. Centrifugation permitted the removal of agglomerated portions, whereupon the change in the suspension was determined by the turbidity of a diluted portion. It was not necessary to add foaming agents, for even in the distilled water there was sufficient foam to observe the effects of entrainment.

Koch bacilli (dried B.C.G.) suspended in water were easily concentrated in the foam and appeared in clumps which adhered to the wall of the tube. The remaining suspension rapidly became more dilute.

Under the same conditions, *B. coli, Staphylococcus alba,* and yeast cells (*Schizosaccharomyces*) were separated in the foam to only a very slight degree. However, by adding NaCl, $Na_2SO_4$, $CaCl_2$, or saponin, mucin or globulins, the separation was greatly enhanced. These effects increased at higher salt concentrations for all the above cases, as well as for Koch bacilli.

On the basis of the above experiments, it was possible to make a nearly complete separation of Koch bacilli from a mixed suspension containing one of the other organisms mentioned previously. A microscopic examination showed no Koch bacilli left in the residual liquid.

Dognon remarks that the separation of Koch bacilli from the other microorganisms in distilled water suspensions appears to be in accord with the concept of microflotation, indicating that the poorly wetted Koch bacilli are entrained in the foam. This may be attributed to the hydrophobic nature of the surface-covering of these bacilli. There is no simple explanation for the effect of electrolytes.

## DISCUSSION

The methods for fractionation in foam which have been summarized suggest procedures which may be useful, but it is evident that no single method is applicable to all cases.

Abribat stresses the importance of taking advantage of differences in foam drainage. On the other hand, in Schütz's apparatus, the foam was removed as soon as possible after formation, so that only a short time was allowed for drainage. Schütz uses "foam time" and the foam volume produced at a fixed gas pressure as a guide to the procedure for fractionation of mixtures of materials in the foam. It appears that "foam time" is affected by drainage rates as well as by surface adsorption, but not either one alone.

It is advisable to separate foam which had been allowed to drain, as much as possible. It may be of interest to speculate with regard to the advantage in foam fractionations of foams with viscous films which drain slowly to a state where relatively large amounts of liquid are retained. Abribat says that, for protein foams, the films are, in effect, gels which are relatively rich in water, whereas, for solid interfacial films, the foam is nearly completely dehydrated when a steady state is reached. In such a case, solid is deposited on the tube or else a rigid fine spongelike structure is formed. It was noted previously that, in our work, a repetition of the operation of pouring the solution through the foam gives improved selective adsorption and a more complete approach to a steady state. All the material which can be removed from solution is not adsorbed in foam immediately. Consequently, slow-draining foams would be expected to lead to better fractionation in Abribat's apparatus, since it is obvious that this results in larger amounts of liquid remaining in the foam (per $cm.^2$) while it is drained, as well as after drainage has practically stopped. In this connection, Schütz made the observation that, the lower the rate of gas flow, the greater the fractionation, due to the longer time allowed for adsorption to reach a steady state.

Abribat hardly mentions adsorption, except to say that it does not determine the fractionation which, he states, is predominantly a function of surface "rigidities." Relative adsorption should be estimated on the basis of the same surface areas, so that cognizance should be taken of the volume of foam and the bubble sizes. In foam fractionation, the total adsorption will, of course, depend on the total surface. In FIGURE 3, taken from Abribat, the curves do not show differences in drainage rates on the basis of comparable total volume of foam, which is proportional to $H + h$, and, furthermore, the various fractions probably correspond to different bubble sizes. These factors, as well as bulk and surface viscosities, affect drainage rates,[13] so that it does not seem valid to attribute the overall differences in drainage rates, indicated by Abribat in FIGURE 3, to differences in "rigidities" of surface films, unless other data are available to show this.

A solution of a mixture of sodium dodecyl sulfate and sodium hexadecyl sulfate does not show rigid films of the type suggested by Abribat. There is no evidence of high surface viscosity in films made from the solution, but a purification of this mixture can be carried out by foam fractionation.[5] In this case, the solution was allowed to flow through the foam several times. The guide to effective separation was obtained on the basis of the surface tension-concentration curve, as described previously.

Gibbs' Equation deals with reversible adsorption, but there is reason to believe that some of the foam fractionations which Abribat and Schütz describe deal with irreversible adsorption of the type due to denaturation of protein.

It would be useful to have more examples of separations of mixtures of relatively pure materials of known composition, in order to learn more with regard to the principles involved. The suggestions given in the papers discussed should be pursued, so that the significant factors leading to clear-cut separations may be more precisely defined. In separations and purifications, it would be desirable to use better criteria of the purity of the fractions obtained.

It is clear that the methods of foam fractionation, purification with emulsions, and microflotation of bacteria can lead to many applications of theoretical and practical importance.

## SUMMARY

Examples of the extent of adsorption at liquid-liquid and air-liquid interfaces have been noted from the literature.

Some of the possibilities of fractionations with foams and emulsions have been reviewed with particular reference to the more recent publications.

The various procedures are described and a number of factors involved are discussed, including selective adsorption, composition and concentration of solution, pH of solution, temperature, size of bubbles, rate of foam formation, eddy currents in inhomogeneous foams, and the effects of bulk and surface viscosity.

The surface tension-concentration and interfacial tension-concentration curves have been shown by Miles and Shedlovsky[4-6] to be a guide to the method for the purification of certain mixtures. Such data are also useful criteria of the purity of some materials.

Abribat[7] and Dognon[14] emphasize that foam drainage is a predominant factor in fractionation. They point out that drainage of foam represents a chromatographic column in which the constituents are stratified according to surface rigidity of the films rather than order of surface activity.

Their drainage data for foams cannot be clearly attributed to relative surface "rigidities", since the variations in surface areas and bubble sizes involved are not taken into account.

Transfer of all the material which can be adsorbed on foam from solution is not instantaneous, and it is suggested that low rates of gas flow and slow-draining foams lead to better fractionations, due to the longer time that foam bubbles are surrounded by adequate amounts of solution.

In the procedure used by Abribat and Dognon, it is claimed that eddy currents of bubbles formed in the foam may be sufficient reason to ruin a separation previously obtained. Abribat describes a device for the agitation of the column of foam which he uses to avoid eddy currents.

It is suggested that the difficulties with regard to eddy currents may be attributed to the formation of foams through fritted diaphragms which do not result in uniform bubble sizes.

Schütz uses "foam time" and foam volume produced at a fixed gas pressure as guides to the procedure for the foam fractionation of mixtures of materials.

Applications are given of foam fractionation of sodium alcohol sulfates, soaps, proteins, collargol, dyes, and bile acids.

The separation, by Dognon, of Koch bacilli from *B. coli, Staphylococcus alba,* and yeast cells (*Schizosaccharomyces*) by microflotation in foam is outlined.

The writer wishes to express his appreciation to Mr. Gilbert D. Miles and Dr. John Ross for their helpful suggestions in connection with this review.

# ION-EXCHANGE ADSORBENTS AS LABORATORY TOOLS

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Ion-exchange adsorbents at present enjoy a wide application in industry. The patent files are rich with examples of production tasks which ion-exchange has ingeniously been made to do. The use of exchange adsorbents as common laboratory tools, however, has yet to receive adequate recognition.

In order to illustrate the broad possibilities for these techniques, it was thought useful to review the principles involved in some of the commercial uses of ion-exchange. An attempt will also be made to discuss typical applications of ion-exchange in organic and biochemical research, in order to make these results known to a wider audience.

Since the concentration, isolation, and purification of organic materials from natural sources or dilute reaction mixtures are prime problems in organic and biochemical research, emphasis will be placed upon the applications of ion-exchange techniques to the accomplishment of these purposes. It will also be seen that ion-exchange provides simple means for the preparation of salts by double decomposition, removal of acid from acid salts, and for the separation of many substances.

While all the techniques reviewed here do not explicitly involve chromatography, it will be seen that they could, in most cases, be applied in the chromatographic manner.

The history of the development of ion-exchangers from the natural clays and humic materials through the synthetic siliceous, the carbonaceous and, finally, the synthetic resin exchangers is recorded in a review by Myers, Eastes and Myers.[63] The theoretical aspects of ion-exchange are discussed by Griessbach[40] and also by Myers in his chapter on synthetic resins in *Advances in Colloid Chemistry*.[61] A more recent discussion is to be found in the paper by L. E. Davis.[28] Studies on rates of exchange have been made by Walton,[86] by Nachod and Wood,[65] and by Thomas.[81] An excellent discussion of the theoretical conditions for exchange reactions is contained in Cannan's paper on "Chromatographic and Ion-Exchange Methods in Amino Acid Analysis".[22] For more complete bibliographies, reference should be made to the general reviews of Block[17] and Deitz.[29]

For the purpose of this discussion, we may consider ion-exchangers to be insoluble acids or bases. As such, they are capable of the reactions of ordinary acids or bases, with the notable exception that one of the reaction products always "leaves the solution" in the form of an insoluble salt. The salt-forming capacities of these insoluble acids and bases are, to a certain extent, a function of the dissociation constants of the reacting electrolytes. Simple exchange reactions may, therefore, be expected to behave in accordance with the mass action law.

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Sussman, Nachod, and Wood,[80] using the case of basic resins as an example, have pointed out that "it is rather difficult to conceive of an ionic system in which the anion is freely mobile in the usual sense while the cation comprises a substantially immobile charged locality on a macromolecular insoluble resin. It is possible that, under these conditions, ionization can consist of little more than a limited mobility of the anions among different cationic groups on the resin."

Since ion-exchange reactions involve a two-phase system, results comparable to those in physical adsorption or extraction by immiscible solvents are obtained. Thus, the rate of a given reaction is influenced as in physical adsorption, by time of contact, surface area, etc. Myers[61] suggested that, in order to determine whether a reaction is one of physical adsorption or of ion-exchange, it is necessary to analyze for all the constituents originally present as well as those which may be formed.

As in the case of "conventional adsorbents", ion-exchangers may be added directly to the solution, and the exchange allowed to reach equilibrium, or they may be used in columns, in which case continuous counter-current effects may be achieved.

In cation exchangers, the exchangeable hydrogen ion may be provided by any one of the following functional groups:

$$\text{—}SO_3H,\ \text{—}CH_2SO_3H,\ \text{—}COOH,\ \text{—}OH,\ \text{—}CH_2OH,\ \text{or}\ \text{—}CH_2SH.$$

The acid-binding properties of anion exchangers may be due to aromatic or aliphatic basic nitrogen groups such as

$$\text{—}NH_2,\ =NH\ \text{or}\ \equiv N.$$

In the ion-exchange reactions listed below, *A* represents an insoluble acidic cation exchanger, and *B* an insoluble basic anion exchanger.

*Base exchange*

$$Na \cdot A + CaCl_2 \rightarrow Ca \cdot A + NaCl \tag{1}$$

*Acid formation*

$$H \cdot A + NaCl \rightarrow Na \cdot A + HCl \tag{2}$$

*Acid removal*

$$B + HCl \rightarrow B \cdot HCl \tag{3}$$

*Anion exchange*

$$B \cdot HCl + NaI \rightarrow B \cdot HI + NaCl \tag{4}$$

The remaining discussion will be organized mainly around these four reactions.

## BASE EXCHANGE

Reaction 1 represents the classical use of zeolite exchangers for the softening of water. In like manner, calcium ions in milk or blood may be exchanged for sodium. In the case of milk, a soft curd milk was produced which was found useful in infant feeding.[55] The treatment of blood with a sodium exchanger was suggested by Steinberg[77] as a means for obtaining "uncoagulated blood."

The quantitative aspects of this exchange have been investigated by Applezweig and Rice,[5] who found that approximately 80 per cent of the blood calcium is removed. Since the amount of calcium which remains is theoretically sufficient to promote clotting, it is assumed that this residual calcium is in a bound or non-ionized form and, as such, not capable of promoting coagulation.

In reaching conclusions concerning the concentration of calcium ion required for the clotting process after the addition of oxalates or citrates, it was necessary to assume certain relationships between the two systems involved, *i.e.,* calcium oxalate or citrate on the one hand and calcium proteinate on the other. Ion-exchange offers a new approach to the study of this mechanism, since calcium is removed simply by exchange with sodium without addition of extraneous anions. Thus, only the natural equilibria in the blood need be considered.

In a like manner, the role of various cations in other physiological phenomena may be explored. It has been generally assumed, for example, that serum calcium is associated with complement function. Ecker and Pillemer[33] studied the effects of calcium "disionizing" anions upon complement and reasoned that calcium plays a role of doubtful significance in complement fixation. In Dr. Ecker's laboratory, human, rabbit, and guinea pig sera which had been treated with a sodium exchanger were found to have lost none of their complement activity.[34] This would seem to confirm the belief that ionic $Ca^{++}$ plays a negligible role. Breazeale, Pierce, and Breazeale,[18] in studies on serological precipitation-flocculation tests, found that the reactivity of sera could be shifted from negative to positive and *vice versa* by treatment with zeolites. These investigators found that Kahn antigen adsorbs "reagin" out of syphilitic serum, thereby producing a seronegative serum. By treating this experimentally produced sero-negative serum with calcium zeolite, they again obtained positive flocculation reactions to the various tests. Later, they were able to apply this same principle to a test for syphilis by employing a suspension of pure sodium zeolite as an indicator.

Pierce and Breazeale[67] then investigated the removal of divalent cations from solutions by beef heart antigens and found Kahn and Hinton antigens to be capable of removing calcium and magnesium from aqueous solution. The floc which is formed by this reaction releases these divalent ions again when it is washed with saline. On the basis of these observations, a new theory of the mechanism of syphilis serology was advanced, namely, that, in positive sera, there must be either an increase in ionization of divalent cations or some profound vectorial shift with respect to the ionization constant of calcium compounds causing the antigen to floc. At any rate, it was shown that beef heart antigens act purely in the capacity of an ion-exchanger and shift from a sodium salt to either a calcium and/or magnesium salt, thereby producing a floc.

An obvious application for base exchange is in the preparation of salts by double decomposition. Thus, the sodium salt of penicillin may be converted to the calcium salt by passage over a calcium exchanger. In some cases, it may be desirable to convert a soluble salt into a less soluble one. This is the principle upon which many of the chromatographic separations using ion-exchange adsorbents are based. The formation of a less soluble salt delays the passage of the compound through the column.

When cations are present as contaminants in crude reaction mixtures, base exchange can be especially useful. For example, the various cationic contaminants may be exchanged for a single one capable of easy removal. Harmful or poisonous cations may be exchanged for harmless ones.

Sugar juices have been commercially purified by means of base-exchange. A natural Ca zeolite was used by Harm, in 1896,[41] to replace molasses-forming sodium and potassium ions in sugar solutions with calcium ions. Jeanprost[49] reported on the treatment of diluted molasses with a Pb-zeolite. Lead salts in solution were later removed by passing the liquid over an ammonium exchanger. Austerweil[9, 10] used $NH_4$-zeolites to reduce substantially the ash content of sugar solutions. $NH_4$ ions which passed into the sugar juice were removed in the form of ammonia by evaporation.

Jones[50] has found that the conversion of calcium and magnesium chlorides in petroleum to sodium chloride by a sodium exchanger results in reduction of corrosion in refinery equipment. Bahrdt[12] has used cation exchange for the removal of interfering metals in an analytical determination of sulfate.

One of the earliest adaptations of base exchange to laboratory problems was that of Folin,[37] who, in 1917, developed a method for the determination of ammonia in urine. Sodium permutit (later called Folin Decalso), is used to "adsorb" the ammonia. After washing, the adsorbent is treated with sodium hydroxide and Nessler's reagent, the ammonia nitrogen being determined colorimetrically.

Ammonia in other fluids may also be determined in this manner. A common way to prepare ammonia-free water is by adding a powdered sodium zeolite.

Bird[15] has used cation exchangers in the preparation of sols of silicic acid and other acidic oxides. According to his patent, relatively pure silica sols are obtained by treatment of a dilute solution of sodium silicate with a hydrogen exchanger. The sodium ion in the silicate solution is replaced by hydrogen to give a silicic acid sol in the effluent:

$$H \cdot A + Na_2SiO_3 \rightarrow Na \cdot A + H_2SiO_3$$

The introduction of carbonaceous and synthetic resinous zeolites capable of hydrogen exchange vastly broadened the possible fields of application for ion-exchangers. These newer-type adsorbents are available as relatively pure compounds possessing reproducible physical and chemical properties. Like the siliceous zeolites, they may be used in sodium or calcium cycles as base exchangers with the advantage that they have a greater exchange capacity per unit volume and are stable over wide ranges of pH.

When used in the hydrogen cycle as shown in REACTION 2, acids are formed. This reaction is especially important when employed together with acid-removing adsorbents (REACTION 3) to effect complete removal of electrolytes from solution. This will be discussed, later, in connection with demineralization.

The exchange of metallic cations for hydrogen offers many other interesting possibilities.

Foremost among these is the possibility of an "acid machine". If a solution containing cations is passed through a hydrogen exchange column, the effluent will be found to contain an amount of acid equivalent to the cations originally present.

Polis and Reinhold[69] have used this principle to determine total base in serum. 0.2 ml. of serum is added dropwise to a micro column of a synthetic hydrogen exchange resin. The column is washed with water and the effluent titrated with standard alkali. Since base which is combined with carbonate or bicarbonate will yield $H_2CO_3$, it is necessary to do a $CO_2$ combining-power determination on another portion of the same serum. The two results, when taken together, represent a value for total base which compares very favorably with that obtained by the more cumbersome method of electrodialysis.

A hydrogen exchanger is made to serve a dual purpose in the manufacture of high-grade pectin.[60] Metallic cation impurities are removed and acid for hydrolysis is provided by the hydrogen exchange. Similarly, the production of a palatable artichoke syrup[35] and the inversion of sucrose solutions may be aided by hydrogen exchangers.

The use of zeolites in the concentration of metal traces offers promise. Little work has been done in this direction. Abrahamczik[1] has shown that by permutitic exchange minute amounts of iron can be isolated and determined. Ten liters of distilled water containing 0.0025 parts per million of ferrous iron were run through a 50 x 450 mm. column of "neopermutit" (about 400 g.). One hundred ml. of warm saturated sodium chloride solution were then passed slowly through the column in the reverse direction, followed by 150 ml. of wash water. Iron was determined colorimetrically in an aliquot of the combined solutions, and 90 per cent of that added was found.

## ADSORPTION OF ORGANIC CATIONS

An observation, in 1921, that adrenaline could be removed from solution by shaking with permutit prompted Whitehorn to investigate the use of this base exchanger as a reagent for amines.[88] He investigated not only what kinds of substances can be removed from solution by base exchange, but also in what ways this reaction can be affected by chemical and physical conditions. The adsorption of fifty-two organic compounds was studied qualitatively, and fundamental facts concerning the quantitative aspects of base exchange were disclosed.

Whitehorn showed that permutit could be used to separate relatively strong nitrogenous bases, having a dissociation constant of $5 \times 10^{-9}$ or greater, from weaker bases and non-basic substances. He found that more of a given base could be removed from neutral solution than from acid or alkaline solution. Bases could also be removed from solution in varying concentrations of ethyl alcohol, in amyl alcohol, and in ether.

For the quantitative removal of bases from solution, it was shown that filtration through the exchanger is preferable to agitation because (1) there is no accumulation of the sodium salt formed by the reaction which would tend to reverse it; (2) successive portions of a relatively large volume of fluid can be rapidly brought into intimate contact with the exchanger; and (3) easily oxidizable substances are saved from destruction.

To liberate the bases from the exchanger, Whitehorn investigated the use of sodium hydroxide, sodium cyanide, and potassium chloride. Of these, he found a saturated aqueous solution of potassium chloride to be the most generally useful reagent. Using this technique, he reported the recovery of 92 per cent of adsorbed ethylamine nitrogen and 85 per cent of adsorbed choline nitrogen.

Later,[90] Whitehorn developed a chemical method for estimating epinephrine in blood using silicic acid as the exchange adsorbent and sulfuric acid as the eluent.

The fundamental observations of Whitehorn provide the necessary background for the use of ion-exchangers in the recovery of organic cations.

One of the most useful applications of this technique has been in the isolation of alkaloids. A typical application is that of Oberst, who used permutit in the determination of morphine in the urine of morphine addicts.[66] Saturated sodium carbonate was used to liberate the alkaloid in the presence of the Folin-Denis phenol reagent. The blue color formed by the reaction of the alkaloid with the reagent was read colorimetrically.

The commercial application of ion-exchange to alkaloid recovery was greatly enhanced by the development of the synthetic hydrogen exchangers. Relatively large amounts of alkaloids could be adsorbed from dilute acidic solutions. Higgins[46] and Riley[72] patented the use of nicotine-containing hydrogen exchangers as horticultural poisons. Tiger and Dean[82] disclosed a procedure for obtaining concentrated solutions of nicotine salts by passage of an aqueous extract of tobacco over a hydrogen exchanger and subsequently liberating the nicotine in the form of its hydrochloride by means of constant boiling hydrochloric acid.

The use of a hydrogen exchanger to adsorb quinine from acid solution and to concentrate the alkaloids in a totaquine preparation was reported by Applezweig.[4] The recovery of the alkaloids from the exchanger was accomplished by means of alkali regeneration and solution in an organic solvent.

This communication also suggested the application of ion-exchange to alkaloid extraction by means of a cyclic system. Such a technique would permit the acid percolate to pass through a column of exchanger and return to the percolator in a continuous fashion. Thus, the drug would be constantly exposed to an extraction fluid free from alkaloids but saturated with respect to non-cationic ingredients.

Based upon these principles, a commercial installation for the isolation of scopolamine from cultivated datura plants was put into operation in the summer of 1944. The procedure proved sufficiently versatile to permit its adaptation to a field process for the manufacture of Cinchona alkaloids from low-quality green bark in jungle areas.[6] A portable unit was designed at the Engineer Board, Fort Belvoir, Va., which is capable of extracting the alkaloids contained in 13,000 lbs. of green bark per month with the use of approximately 1,000 lbs. of chemicals. The losses and much of the expense involved in the drying, grinding, and shipping of Cinchona bark to extraction plants may thus be eliminated.

The findings of Whitehorn that organic bases of moderate strength could be removed from solution by base exchange suggested to Cerecedo and Hennessy the idea of using zeolites for the isolation of vitamin $B_1$.[25] Extracts of rice polishings, brewer's yeast, and wheat germ were passed hot over a bed of Decalso (a synthetic siliceous zeolite) which had previously been stirred with sulfuric acid at a pH of 4. The adsorbed vitamin was then released by exchange with ammonium ion from a hot molar solution of $NH_4NO_3$. A single silver precipitation, followed by a precipitation with silicotungstic acid, yielded highly potent concentrates, from which, on recrystallization, crystals of pure vitamin hydrochloride could be obtained.

The successful commercial synthesis of thiamin precluded the wide use of base exchange for the recovery of this compound from natural sources, but the method of Cerecedo and Hennessy has become a standard one for the determination of vitamin $B_1$ in foodstuffs and physiological fluids.[43, 44]

Recently, Herr[45] studied the use of synthetic cation exchange resins in the separation, recovery, and concentration of thiamin. It was found that the acid-regenerated form of the resin completely removes thiamin, for which the resin has a large capacity, in the presence of riboflavin. Since riboflavin is not adsorbed under the conditions in which thiamin is completely removed, the separation is satisfactory. Thiamin is eluted in excellent yields by passage of strong mineral acid through the column.

The affinity of riboflavin for the resinous hydrogen exchanger was found to be much less than that of thiamin. Also, elution of riboflavin could be accomplished with a lower concentration of acid than that needed for thiamin recovery.

The purity of the eluate was found by Herr to be satisfactory for thiochrome analysis. For the recovery of thiamin from natural sources, the resin offers a distinct potential advantage over siliceous zeolite as an adsorbent; namely, elution can be accomplished with a volatile acid instead of a salt which cannot be removed readily from the eluate.

Although Binkley and co-workers have reported on the use of a Decalso column in the isolation of vitamin K,[14] in this case the adsorbent did not serve as an ion exchanger. The vitamin was removed from a petroleum ether solution and eluted by means of benzene in petroleum ether or acetone.

In 1932, Lejwa[53] purified the gonadotropic hormone by shaking urine with permutit for 2 hours and then eluting the adsorbed hormone with dilute $NH_4OH$. Active crystalline material was thus obtained which assayed 1,000 mouse units per mg.

Katzman *et al.*[51] were able to prepare concentrates of the hormone possessing 8,500 I.U. per mg. by adsorption on permutit and elution with an alcoholic solution of ammonium acetate. Adsorption from acidified pregnancy urine was found to take place at pH 4, but very little hormone was adsorbed at pH 5. If the hormone functions as a cation in the ionic exchange, then this would indicate that pH 4 is below its isoelectric point. Since evidence by other investigators places the isoelectric point between pH 3 and 3.5, the authors admit that it is possible that some other physical phenomenon is responsible for the adsorption.

Potts and Gallagher[70] used "Folin permutit" to separate, in high yield, the oxytocic and pressor principles of pituitary extract. The pressor hormone, a more basic ampholyte than the oxytocic principle, is adsorbed on the base exchanger. The separated principles offer a marked advantage for the further purification of the two products, since the losses are slight and the separation of the two activities is fairly complete. The pressor principle was adsorbed from aqueous solution and eluted with 10 per cent sodium chloride solution.

The adsorption of the active principle of hypertensin upon a hydrogen exchange resin has been announced by Cruz Coke, Gonzalez, and Hulsen.[27] No effective elution procedure was, however, found by these investigators.

A method for the determination of citrulline and allantoin based upon the cation exchange removal of the former from serum containing both compounds was described by Archibald, in 1944.[7] In developing this method, Archibald investigated quantitatively the adsorption by ZeoKarb and Amberlite IR-100 of citrulline, allantoin, allantoic acid, alloxanic acid, alloxan, alloxantin, parabanic acid, methylurea, phenylurea, and thymol.

McColloch and Kertesz[58] reported separation of pectin-methylesterase and pectin-polygalacturonase from a commercial pectinase by the action of a cation exchange resin. The authors explained their results in the following way:

The removal of pectin-methylesterase, but not of pectin-polygalacturonase, will be accomplished where the pH of the solution is below the isoelectric point of the methylesterase but still above that of the polygalacturonase. The authors had observed the different behavior of these two enzymes towards acids, and the fact that they had different pH optima made it likely that they would behave differently on ion exchange columns. They believe that the method will prove applicable to the separation of some other enzymes.

Among the bases which Whitehorn found removable from solution by permutit, were the basic amino acids, arginine, histidine, and lysine. Dubnoff[32] applied this finding to the separation of arginine from a protein hydrolysate for subsequent estimation by the Sakaguchi reaction.

Archibald was able to prepare glutamine solution free of contaminating arginine by passage of the contaminated solution through a bed of Decalso.[8] The effluent contained 90 per cent of the glutamine, but no arginine. The column was then washed with water to yield all of the glutamine left in the column and 25 per cent of the adsorbed arginine. A final washing with 3 per cent sodium chloride yielded the remaining 75 per cent of adsorbed arginine and no glutamine.

The use of strong neutral salt solution for the removal of the basic amino acids from siliceous sodium zeolites interferes seriously with subsequent isolation and purification steps. Block, in 1942, developed a method for the separation of basic amino acids from the other constituents of protein hydrolysates based on the use of hydrogen exchange resins.[16, 17] The basic amino acids are recovered in the form of a concentrate by treating the adsorbates with 7 per cent HCl.

At about this time, a number of workers were investigating the possibility of using ion exchange to sort amino acids into three groups: the acidic amino acids, held by the acid-binding resins; the basic amino acids by cation exchange; and the essentially neutral amino acids which might reasonably be expected not to be held by exchange adsorbents. Using synthetic resin exchangers, Freudenberg, Walch, and Molter[38] found that all amino acids could be taken up by a cation exchanger and preferential elution would have to be used in recovery of different fractions. Tryptophane was specifically separated from other amino acids by Turba, Richter, and Kuchar, using an organic cation exchanger.[85]

Englis and Fiess[36] and, later, Cleaver, Hardy, and Cassidy[26] reported on the conduct of amino acids in various exchange reactions. Their work confirmed the fact that hydrogen exchangers were capable of adsorbing all amino acids to some extent.

The latter investigators explain this seeming deviation from the hypothetical by the fact that the acidifying action at the surface of the resin brings all amino acids at least partially into the cation form and thus makes them "exchangeable". Also, "onium" formation may occur at the surface of the resin, converting a strongly acid resin, strongly acid due to sulfonic acid groups, into a less strongly acid adsorbate-resin, acid now due to carboxylic groups. The ammonium and sodium forms of the resins, on the other hand, were found by Cleaver, Hardy, and Cassidy to be quite selective for the cationic amino acids.

These authors also discussed their data with a view to the use of ion exchangers for chromatographic separations. They predict the possibility of separating arginine and histidine in this manner, since they have different adsorption isotherm slopes.

Sims[75] has improved on the Dubnoff determination of arginine by chromatographically separating it from glycocyamine. Both compounds give the Sakaguchi reaction. By careful control of the flow rate and salt concentration in a sodium resin exchange cycle, the glycocyamine is successively passed down the column and eluted in advance of the arginine.

## ACID ADSORPTION AND DEMINERALIZATION

Since whole molecules of acid are adsorbed by the amine-type resins, it may be said that these exchangers really act as acid adsorbents (REACTION 3). Acids so adsorbed may be recovered by displacement with another acid, or in the form of a salt by regeneration of the exchanger with an alkali.

Unlike the base exchangers, there were no anion exchangers in common use as adsorbents prior to the advent of synthetic resinous exchangers.

The researches of Adams and Holmes, in 1935,[2] and of Griessbach, in 1939,[39] made possible the production of basic resinous adsorbents. Since these adsorbents have been commercially available, many possibilities for these valuable tools have been realized.

As has been mentioned previously, the use of acid-binding resins for the removal of acidic amino acids is an important one. When Block[16] isolated basic amino acids by means of a hydrogen exchange resin, he first pre-treated the protein hydrolysate to remove excess acid. This was accomplished by vacuum evaporation and finally by stirring with acid-binding resin until the reaction of the solution was approximately pH 6. In this same year, Freudenberg, Walch, and Molter[38] showed that acidic amino acids were adsorbed preferentially by an acid-binding resin. Cannan[21] used a basic resin for the separation of the dicarboxylic amino acids from protein hydrolysates. Estimations of the glutamic acid and aspartic acid in egg albumin, B-lactoglobulin, and edestin were reported. The adsorbed dicarboxylic acids are eluted, according to Cannan's procedure, with 0.25 M hydrochloric acid. The amino acids in the eluate are readily crystallizable, or they may be estimated by determination of amino nitrogen and carboxylic values, since the resin was found to be remarkably selective for the dicarboxylic acids.

The observations of Cannan were confirmed and the quantitative aspects of the anion exchange of amino acids studied by Englis and Fiess[36] and Cleaver, Hardy, and Cassidy.[26] The latter group concludes that much work remains to be done before resins can be used with complete confidence in the analytical separation of amino acids, but they offer many stimulating suggestions regarding new techniques which should be tried.

A neat illustration of one of the many ways in which acid-binding resins can be used is that of Buc, Ford, and Wise.[19] This group, while working upon an improved synthesis of $\beta$-alanine, was faced with the problem of liberating $\beta$-alanine from its hydrochloride. The use of lead oxide for this purpose was found to be tedious and troublesome. Attempts to use aniline or pyridine in methanol were unsuccessful. A method using pinene for this purpose was reported by Austin[11] to give only a 50 per cent yield. Buc, Ford, and Wise found that, when an aqueous solution of pure $\beta$-alanine hydrochloride is passed through a bed of the acid-binding resin, the resulting effluent is substantially chloride-free. The yield of chloride-free $\beta$-alanine is 93 per cent when pure $\beta$-alanine hydrochloride is used, or 83-88 per cent when the crude hydrochloride is used. Since the monoaminomonocarboxylic and the basic amino acids are not adsorbed by acid-binding resins, this method would prove generally useful for obtaining other amino acids from their anion salts.

When REACTION 3 (acid adsorption) is used in combination with REACTION 2 (hydrogen exchange) the technique referred to as *demineralization* or *deionization* becomes possible. Thus, when a salt solution is passed through a tandem setup, first through a hydrogen exchanger and then through an acid adsorbent, an acid is formed in column one where the cation has been exchanged for hydrogen, and the acid is removed in column two, thus removing the anion and yielding a solution free from electrolytes.

The possibilities for demineralization were immediately realized by the commercial water-conditioning organizations, whose engineering staffs have now made possible the production, at very low cost, of demineralized water which is said to compare favorably with that obtained previously by distillation.[42, 83]

Liebig, Vanselow, and Chapman[54] have pointed out the suitability of water purified by ion exchange resins for the caring of plants in controlled nutrient cultures. Objectionable traces of copper and other heavy metals which may occur in distilled water are absent from ion exchange water.

The removal of ionic contaminants from solutions of non-electrolytes should be of considerable interest to the organic chemist and biochemist. Smit[76] proposed the demineralization of solutions of non-electrolytes by passage over ion exchange adsorbents. His patent describes the purification of glucose solutions, paraffin, and other impure liquids, by treatment with a hydrogen exchanger and subsequently removing the free acid by means of an acid adsorbent.

Another application of ion exchange for the complete demineralization of non-electrolyte solutions was that of Holmes[48] who purified gelatin solutions by passage alternately over a bed of cation and anion exchange adsorbent.

Rawlings and Shafer, in 1942,[71] reported on the demineralization of clarified sugar cane juice. They found that, in a commercial process, cation and anion exchangers removed 95 per cent of the ash, 32 to 50 per cent of the original impurities, and 50 per cent of the color. A similar process for the purification of beet juice was described by Weitz, in 1943.[87] Applying such a procedure in the laboratory, Platt and Glock[68] used demineralization to purify an extract obtained from muscle preliminary to the estimation of the inositol content. By this treatment, the inositol-containing solution was obtained in a purer condition than by the conventional precipitation procedures previously used.

Williams and Johnson[90] have devised an improved technique for the determination of pectin. They found that pectin can be quantitatively determined by electrodeposition, provided the electrolyte concentration of the solution is low. Ion exchange resins were used to effect this lowering of electrolyte concentration. Here, again, is a method of broad possibilities: the isolation of electrically charged colloids by electrodeposition from solutions which have been "deashed" by ion exchange.

Tandem column operation is often useful where the desired ingredient of a mixture is exchangeable. McCready and Hassid[59] found that the preparation of the Cori ester from starch by Hanes' procedure can be very much shortened by using ion exchange adsorbents. Following phosphorolysis of the starch in the presence of phosphate buffer and the precipitation of inorganic phosphate, the reaction mixture was first passed through a column of hydrogen exchange adsorbent, which removed cations from solution. The effluent was then run through an acid-adsorbing column, where the glucose 1-phosphate, being a strong acid with high exchange capacity, was adsorbed. Soluble impurities including dextrins, proteins, and weak organic acids passed through unchanged. The glucose 1-phosphate was then eluted from the adsorbent with dilute alkali and isolated as the crystalline dipotassium salt.

Since cation exchangers had been used in the preparation of sols of acidic oxides,[15] Ryznar used anion exchangers for the preparation of hydrous oxide sols.[73] An alkali-regenerated anion exchanger was used in the preparation of sols of hydrous aluminum oxides, ferric oxides, and other metal oxides.

If the alkali-regenerated anion exchanger is considered to act only as an acid-binding agent, the reactions may be expressed as follows:

(1) $2AlCl_3 + 6H_2O \rightleftharpoons 6HCl + Al_2O_3 + 3H_2O$

(2) $6B + 6HCl \rightarrow 6B \cdot HCl$

$SO_4^{--}$, $Cl^-$, $NO_3^-$, and other anions may be removed in this way. The process is most successful when very dilute solutions are used. When concentrations of more than 1 per cent are used, aluminum and ferric sols show a tendency to precipitate and clog the bed.

Ryznar further demonstrated that a combination of cation and anion exchangers could be used to purify colloids formed by other methods. Colloidal sols formed by double decomposition or hydrolysis can be passed through the two exchange beds in series with a resultant removal of most of the electrolytes. This method was found to be simpler and quicker than the use of dialysis for the same purpose.

The preparation and properties of a protein-formaldehyde acid-binding resin were reported by Bhatnagar and associates.[13]

Adsorption experiments with fumaric, maleic, and citraconic acids showed that with $NH_3$-condensed and amino resins the adsorption of *cis* acid is greater than that of *trans* acid. This paper also discussed the adsorption of oxalic, malonic, succinic, aconitic, mono-, di- and trichloracetic, cyanacetic, hydroxyacetic, aminoacetic, propionic, chloropropionic, lactic, amino-propionic, pyruvic, levulinic, dibromosuccinic, malic, tartaric, aminosuccinic, and citric acids.

When it is desired to isolate organic acids which are in solution in the form of their salts, the demineralization procedure may also be resorted to. The recovery of tartrates from grape wastes by ion exchange was studied by Matchett *et al.*[57] Perhaps 10 million pounds per year of tartaric acid are potentially available from the waste pomace and still slops of the grape juice pressing and wine and brandy making industries. The tartaric acid, which is present mostly as potassium bitartrate, is first converted to the free acid by passage through a hydrogen exchanger. The acid is then adsorbed on an anion exchanger from which it may be released by regeneration with sodium carbonate. A solution of the sodium salt is obtained which is from ten to twenty times as concentrated as the original slop and substantially free from the many impurities that would accompany it through a similar evaporative concentration. In the final step, calcium chloride is added to precipitate the tartrate in the form of a relatively pure calcium salt.

## ANION EXCHANGE

In their study of tartrate recovery, Matchett and his group made several interesting observations regarding anion-exchange phenomena. When solutions of potassium bitartrate were passed over anion exchangers which had been regenerated with sodium hydroxide, tartrate ion was fixed to an extent equivalent to precisely half of the total present, while the other half passed through as the normal salt. When a sodium carbonate regenerated anion exchanger was used, a larger amount of tartrate was removed and an equivalent quantity of carbonate ion appeared in the effluent.

From this, the authors infer that both regeneration and loading are definite anion-exchange phenomena. "Beds regenerated with carbonate retain an equilibrium load of carbonate ion, and this can be replaced under the conditions outlined by tartrate. Beds regenerated with caustic alkali solutions would not retain anions and acid adsorption would proceed through fixation of hydrogen ion with consequent retention of an equivalent anion."

Further experiments by this group have shown that it is possible to set up a definite replacement series of anions and that the course of exchange will be fixed by the relative positions of the ions involved and their activity in the solution. For example, tartrates may be recoverable from *unacidified* still slop by exchange with chloride on an anion exchanger regenerated by means of a sodium chloride solution.

In a similar study, Buck and Mottern[20] attempted the purification of apple syrup by ion exchange. It was hoped that ion exchange would remove malic acid and also reduce the lead and arsenic content. It was found that about 80 to 90 per cent of the acid is removed and a palatable syrup is produced by treatment with a single anion exchanger. A variable amount of arsenic is removed by the single treatment. To ensure a more complete removal when it becomes necessary, a two-step (cation-anion) or a three-step (anion-cation-anion) exchanger treatment must be used. As much as 96 per cent of the arsenic has been removed by the three-step method, and a smaller amount has been eliminated by the two-step method. In addition, the cation exchanger removes about 90 per cent of the ash.

Additional evidence for the use of basic resins as anion exchangers has been provided by the work of Sussman, Nachod, and Wood[80] on metal recovery by anion exchange. Metals which form complex ions may be recovered by true anion exchange. Use was made of the fact that treatment of an anion exchanger salt with a solution of a salt whose anion has a higher valence or weight than that of the anion already adsorbed on the anion exchanger, results in the displacement of the adsorbed anion by that of the salt in the influent solutions. Using chromium as an example, best results were obtained with adsorption from a neutral chromate solution on the exchanger chloride. Alkaline regeneration yielded a concentration of the complex ion in the effluent which was more than twenty times that in the influent.

Certainly a most important anion is that of penicillin. While it is known that the use of ion exchangers in the isolation and purification of penicillin has been thoroughly investigated, this information has not yet been made public. A short note in *Science,* in 1944, reported that a South American group had used ion exchange resins for the purification of crude culture filtrates containing penicillin.[27] The filtrate so treated retained all of its antibiotic activity but was free of toxicity. No details of value were, however, given.

Carter *et al.,*[24] in a paper on *Isolation and Purification of Streptomycin,* describe a chromatographic procedure which is essentially one of ion exchange. "Alkaline alumina removes streptomycin from neutral aqueous solutions, and elution with aqueous acid is slow and incomplete. Acid-washed alumina does not remove streptomycin from an aqueous solution but does so from aqueous methanol. This information provided the basis for a chromatographic method of purification. If a faintly acid solution of crude streptomycin chloride in 70 to 80 per cent methanol is percolated over a sulfuric acid-washed alumina column (pH 5 to 6), an inactive fraction first appears followed by an active fraction. The active material contains sulfate ion but no chloride. Evidently chloride ion has been replaced by sulfate from the column and the streptomycin sulfate thus formed then passes through the column less rapidly since the sulfate is much less soluble than the chloride in methanol."

## ION EXCHANGE AND CHROMATOGRAPHIC TECHNIQUES

Because synthetic exchangers have a large capacity and react in a predictable fashion, their use in procedures such as the one noted above for streptomycin appears inviting. Thus, by exchange, less soluble salts may be formed which delay the passage of the compound through the column.

Another variation in technique is possible in the case of compounds such as the alkaloids. Here, adsorption takes place from aqueous solution by exchange with hydrogen ion in a cation exchanger. Elution, however, requires that the adsorbent be made alkaline and then leached with a solvent. The alkaloids may be separated by collection of portions of the eluate in the manner of the typical liquid chromatogram.

Direct visual observation of chromatographic banding on synthetic resins has been reported by Myers, Eastes, and Urquhart.[64] Light orange anion exchange resins were noticed to form a reddish-orange band superimposed on a brown band when a mixture of hydrochloric and sulfuric acids was flowed through the column of the resin. Analysis revealed that the reddish-orange band was the pure hydrosulfate, the brown band the pure hydrochloride form of the resin.

Even on darkly-colored cation exchange adsorbents, adsorption may be followed visually by the use of ultraviolet light. The adsorption of quinine upon sulfonated coal and its subsequent elution has thus been observed by the author.

The use of light-colored resins treated with indicators and of finely powdered exchangers in classical chromatographic techniques is an inviting field for discovery. Even more promising are the prospects for their

APPENDIX: LIST OF COMMERCIALLY AVAILABLE ION-EXCHANGE ADSORBENTS

| Trade name | Manufacturer* | Type | Composition | Capacity,† gram milli-equivalents/ml. of exchanger |
|---|---|---|---|---|
| Amberlite IR-100 | 5 | Synthetic resin cation exchanger | Modified phenol formaldehyde-sulfonic | 0.44 @ 1.38 gm. m. eq. regenerant/ml. of exchanges |
| Amberlite IR-4B | 5 | Synthetic resin acid adsorbent or anion exchanger | Modified phenol formaldehyde polyamine condensate | 1. HCl—1.21 @ 1.66 gm. m. eq. $Na_2CO_3$ as regenerant<br>2. $H_2SO_4$—1.54 @ 1.66 gm. m. eq. $Na_2CO_3$ as regenerant |
| De-Acidite | 4 | Synthetic resin acid adsorbent or anion exchanger | Aliphatic amine resin | 0.55 to 1.2 |
| Decalso | 4 | Synthetic sodium aluminum silicate cation exchanger | $Na_2O:Al_2O_3:6\ SiO_2$ | 0.40 to 0.55 |
| Duolite $A_1$ | 2 | Synthetic resin acid adsorbents or anion exchangers | | 1.68 to 1.8 |
| $A_2$ | | | | 2.0 to 2.2 |
| $A_3$ | | | | 1.4 to 1.7 |
| Duolite $C_1$ | 2 | Synthetic resin cation exchangers | | 0.5 to 0.55 |
| $C_2$ | | | | 1.115 to 1.25 |
| Ionac A-293-M | 1 | Synthetic resin acid adsorbent or anion exchanger | Stable polymer with active amine groups | 0.75 (breakthrough) |
| Ionac A-300 | 1 | Synthetic resin acid adsorbent or anion exchanger | Stable polymer with active amine groups | $H_2SO_4$—1.5 (breakthrough) |
| Ionac C-200 | 1 | Synthetic resin cation exchanger | Stable polymer with active $SO_3H$ groups | 0.55 (breakthrough)<br>0.82 total |
| Nalcite—MX | 3 | Synthetic resin cation exchanger | Modified phenol formaldehyde resin with nuclear $SO_3H$ groups | 0.52 to 0.70 |
| Zeo-Dur | 4 | Processed glauconite | $K(Fe, Al)Si_2O_6$ | 0.125—0.150 |
| Zeo-Karb | 4 | Coal derivative cation exchanger | Sulfonated coal | 0.320—0.550 |

* 1 = American Cyanamid Company, New York, N. Y. 2 = Chemical Process Co., San Francisco, Calif. 3 = National Aluminate Corp., Chicago, Ill. 4 = The Permutit Co., New York, N. Y. 5 = The Resinous Products & Chemical Co., Philadelphia, Pa.

† The actual capacity will depend upon: (1) Regenerant. (2) Regenerant concentration. (3) Regenerant amount. (4) Rate of regeneration, or time of contact. (5) Direction of regeneration as compared with adsorption run. (6) Total solids in liquor treated. (7) Nature of ions present and amounts. (8) Rate of flow of liquor treated. (9) Depth of adsorbent bed.

application in the quantitative analytical techniques described at this conference by Dr. Claesson and Drs. Moore and Stein. Preliminary experiments performed by the author in the laboratory of Dr. K. G. Stern, utilizing exchange adsorbents as retardation barriers in the frontal analysis technique of Tiselius,[84] indicate that the "predictable" properties of ion exchangers may be put to good use.

The techniques of displacement analysis and even partition chromatography may be expected to yield highly interesting results with ion exchangers. As new exchange adsorbents become available through synthesis, the horizons for ion exchange techniques may be expected to broaden and further to enrich the contributions of chromatography to chemical research.

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# THE SURFACE AREAS OF SOME SOLID ADSORBENTS OF POSSIBLE USE IN CHROMATOGRAPHY

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Most of the adsorbents available to research workers in chromatography are, naturally, those intended for industrial use. One problem with which all manufacturers of commercial adsorbents have to contend is the reproducibility of the product. Modern control methods are tending towards a more uniform product, but it is a fact that fluctuations do exist in the adsorptive properties of various consignments. Consequently, a research laboratory which plans to undertake a modest investigation using some one adsorbent, can best serve its future needs by purchasing about one hundred pounds of the adsorbent, mixing it well, and placing this stock in storage. Two-gallon narrow-neck rubber-stoppered glass bottles make convenient storage containers for laboratory work.

Since the commercial adsorbents are intended for industrial application, they are designed for use either in filtration through columns or in a batch process by agitation with the solution which requires subsequent filtration. This situation limits the available particle sizes. Practical aspects dictate that the particle sizes for the adsorbents used in column filtration lie in the range between 4-mesh and 40-mesh. Uniformity in particle size has not been stressed as much as it should be. In the batch process, the manufacturer would like to use as small a particle size as possible, since, thus, the adsorbent appears to best advantage in commercial application. However, the necessary subsequent filtration operation usually places a limit on the amount of very small particles that may be added. Too fine a particle size would consume too much filter aid in order to obtain the required filtration rates.

The commercial solid adsorbents of the United States of possible use in chromatography are listed in TABLE 1. The division into four groups is not claimed to be unique. Also, no claim is made for completeness. The choice of an adsorbent for use in chromatography is made primarily on an empirical basis and great ingenuity has been exercised by the various investigators in matching the adsorbent or mixture of adsorbents to a particular problem.

It has been pointed out by Drake and Ritter[1] that porous materials are characterized by two related qualities: first, a particle density which is appreciably lower than the true density of the material, and, second, a surface area which is greater than the observable geometric surface area.

TABLE 1

SOLID ADSORBENTS IN THE UNITED STATES FOR POSSIBLE USE IN CHROMATOGRAPHY*

| Name | Manufacturer |
|---|---|
| *A: Carbon Adsorbents* | |
| 1. Activated Carbons from coal | Pittsburgh Coke and Chemical Company, Pittsburgh, Pa. |
| 2. Blood charcoal, acid washed | Eimer and Amend, New York, N. Y. |
| 3. Bone Char from the pyrolysis of animal bones | American Agricultural Chemical Company, Detroit, Mich.<br>Baugh and Sons Company, Philadelphia, Pa.<br>Consolidated Chemical Industries, New York, N. Y. |
| 4. "Cliffchar" from wood charcoal | Cliffs-Dow Chemical Company, Marquette, Mich. |
| 5. Columbia Activated Carbons from coconut shells and hard nuts | Carbide & Carbon Chemicals Corporation, New York, N. Y. |
| 6. "Darco" Carbons from lignite or wood | Darco Corporation, New York, N. Y. |
| 7. "Minchar" from carbonaceous slate | Minchar Manufacturing Co., Elmira, N. Y. |
| 8. "Norit" Carbons from pinewood-charcoal | American Norit Company, Inc., Jacksonville, Fla. |
| 9. "Nuchar" Carbons from paper-pulp waste liquors | West Virginia Pulp & Paper Company, New York, N. Y. |
| 10. "Suchar" from paper-pulp waste liquors | West Virginia Pulp & Paper Company, New York, N. Y. |
| 11. Sugar Charcoals | Laboratory Supply Firms |
| 12. "Weschar" from lignite | Western Filter Company, Denver, Colorado |
| *B: Mineral Adsorbents* | |
| 1. Activated Alumina from bauxite ores | Aluminum Ore Company, East St. Louis, Ill. |
| 2. "Alba-floc", a finely divided $CaSO_4$ | U. S. Gypsum Company, Chicago, Ill. |
| 3. Attapulgus clay products from clay deposits | Attapulgus Clay Company, Philadelphia, Pa. |
| "Driocel", from bauxite | |
| 4. Bleaching Clay number 260 | Industrial Minerals & Chemical Co., Berkeley, Calif. |
| 5. "Celite" Filter Aids, diatomaceous earths | Johns Manville Corporation, New York, N. Y. |
| 6. "Crystalite", a zeolite for water softening | Infilco, Inc., Chicago, Ill. |
| 7. "Dicalite" Filter aids, diatomaceous earths | Dicalite Company, New York, N. Y. |

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* A more comprehensive tabulation may be found in: **Victor R. Deitz.** *Bibliography of Solid Adsorbents.* 958 pp. (J. M. Brown, Committee Chairman, Revere Sugar Refinery, 333 Medford St., Charlestown 29, Mass. 1944).

TABLE 1 (*continued*)

| Name | Manufacturer |
|---|---|
| 8. Filtrol Products:<br>"Filtrol", an activated clay from bentonites of montmorillonite structure<br>"J-Neutrol", same as "Filtrol"<br>"Desiccite", a dehydrated bentonite | Filtrol Corporation, Los Angeles, Calif. |
| 9. Floridin Products, bleaching earths:<br>"Diluex", a selected "Floridin" of which 85% is finer than 300 mesh<br>"Floraid", an adsorbent filter aid—95% 300 mesh<br>Florida Fullers Earth, one grade called "Floridin", a natural fullers earth and another called "Florex", an extruded fullers earth*<br>"Florigel", a hydrated Florida Fullers Earth†<br>"Florisil", an analytical adsorbent 40-60, 60-100 mesh, also from 4 to 300 mesh<br>"Florite", an activated bauxite in 20-60 and other meshes | Floridin Company, Inc., Warren, Pa. |
| 10. Gypsum, hydrated | Chemical Supply Firms |
| 11. Lloyd's reagent, prepared from fullers earth | Eli Lilly & Company, Indianapolis, Ind. |
| 12. "Porocel", an activated bauxite | Porocel Corporation, Philadelphia, Pa. |
| 13. "Sinclair Earth", a fullers earth | Sinclair Refining Company, New York, N. Y. |
| 14. Talc, purified powder U.S.P. | Eimer and Amend, New York, N. Y. |
| 15. "Volclay", a natural bentonite | American Colloid Co., Chicago, Ill. |
| 16. "Zeo-Dur", the zeolitic green sand | Permutit Company, New York, N. Y. |

*C: Inorganic Compounds*

| | |
|---|---|
| 1. Aluminum Oxide, anhydrous (purissimum or according to Brockman) | Merck & Company, Inc., Rahway, N. J. |
| 2. Calcium hydroxide (lime) | Chemical Supply Firms |
| 3. Calcium carbonate, precipitated | Chemical Supply Firms |
| 4. "Decalso", synthetic zeolite | Permutit Company, New York, N. Y. |
| 5. "Defluorite", a tricalcium phosphate preparation | Aluminum Corporation of America, Pittsburgh, Pa. |
| 6. "Drierite", anhydrous $CaSO_4$ | W. A. Hammond Drierite Company, Xenia, Ohio |
| 7. "Hydralo", an activated aluminum oxide | J. T. Baker Chemical Company, Phillipsburg, N. J. |
| 8. Magnesia | Chemical Supply Firms |

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* "B" 16-30 mesh; "S" 30-60 mesh; "XXS" 60-100 mesh; "XXF" 90% through 100 mesh; "XXX" 90% through 200 mesh.

† On 70 mesh 4%; 70-80, 4%; 80-100, 5%; 100-140, 14%; 140-200, 11%; 200-300, 14%; through 300, 48%.

TABLE 1 (*continued*)

| Name | Manufacturer |
|---|---|
| 9. (Micron Brand Magnesium oxide, #2641) | Westvaco Chlorine Products Corp., New York, N. Y. |
| 10. Magnesium trisilicate "Magnesol" | Westvaco Chlorine Products Corp., New York, N. Y. |
| 11. Magnesium trisilicate, "Florisil" | Floridin Company, Inc., Warren, Pa. |
| 12. Magnesium trisilicate No. 34 | Philadelphia Quartz Company, Ltd., Berkeley, Calif. |
| 13. "Santocel", a silica aerogel | Monsanto Chemical Company, Boston, Mass. |
| 14. "Silene EF", a synthetic, hydrated calcium acid silicate | Columbia Chemical Division, Pittsburgh Plate Glass Company, Barberton, Ohio |
| 15. Silica gel "Protex-Sorb" | Davison Chemical Company, Baltimore, Md. |

*D: Organic Compounds*

| | |
|---|---|
| 1. "Amberlite" ion-exchange resins:<br>IR—1, IR—100 cation exchangers (phenol-formaldehyde)<br>IR-4, acid-adsorbent (amine-formaldehyde) | Resinous Products & Chemical Co., Philadelphia, Pa. |
| 2. Cellulose, many sources, one being filter paper shredded in a Waring Blendor | |
| 3. "Deacidite", ion exchange resin | The Permutit Company, New York, N. Y. |
| 4. "Duolites", ion exchange resins | Chemical Process Company, San Francisco, Calif. |
| 5. Inulin | Eimer and Amend, New York, N. Y. |
| 6. "Ionac", ion exchange resins, made from melamine compounds | American Cyanamide & Chemical Co., New York, N. Y. |
| 7. Lactose | Eimer and Amend, New York, N. Y. |
| 8. Sucrose—multiple X Confectioners' powdered sugar with 3% starch | Cane Sugar Refineries |
| 9. "Zeo-Karb", a carbonaceous zeolite from coal | The Permutit Company, New York, N. Y. |

This observation is true for most solid adsorbents since they, too, may be rather porous substances.

It has been amply demonstrated in a great number of recent investigations that the extent of surface is the factor of decisive importance in physical adsorption. In chemisorption, and in adsorption from solutions, the extent of surface is an important auxiliary factor in determining the progress of a chemical interaction of the surface with the adsorbate. Previous to the work of Brunauer, Emmett, and Teller,[24] the extent of surface could be determined, by the then available methods, only with an accuracy corresponding to a 2- to 4-fold order of magnitude. It is now possible, by means of the determination of adsorption of nitrogen at low temperatures, to ascertain the surface area with an absolute accuracy of

TABLE 2

SURFACE AREA AND DENSITY OF SOME SOLID ADSORBENTS

| Material | Surface area | | Density gms./ml. | | | Reference |
|---|---|---|---|---|---|---|
| | m.$^2$/gm. | Gas at °C. | Bulk | Particle | True | |
| *A: Carbon Adsorbents* | | | | | | |
| Bone char, new | 120 | $N_2$ at-195° | 0.8 | — | 2.88 | 2, 7 |
| Coconut-shell charcoal | 1700 | “ | — | — | 2.08 | 2 |
| “Darco G 60” | 1300 | “ | — | — | — | 2 |
| “Darco S 51” | 500 | “ | — | — | — | 2 |
| Granular “Darco” | 620 | “ | — | — | 2.14 | 3 |
| “Darco” carbon | 560 | “ | 0.36 | 0.732 | 2.086 | 1 |
| “Columbia” carbon | 1397 | “ | 0.42 | 0.754 | 1.897 | 1 |
| Graphite, powdered | 30.73 | “ | — | — | 2.26 | 4, 7 |
| Carbon blacks: (see also 26) | | | | | | |
| “Arrow black” (.03 μ) | 112 | “ | — | — | — | 2, 4 |
| “Acetylene black” (.05 μ) | 64.5 | “ | — | — | — | 4 |
| “Micronex” (.03 μ) | 106.7 | “ | — | — | — | 4 |
| “Thermatomic carbon” (.5 μ) | 6.81 | “ | — | — | — | 4 |
| “Norit” | 930 | “ | — | — | 2.04 | 2, 7 |
| “Suchar” | 850 | “ | — | — | — | 2 |
| Petroleum coke | 0.52 | “ | — | — | — | 2 |
| “Zeo-carb” | 0.2 | “ | — | — | — | 7 |
| *B: Mineral and Inorganic Adsorbents* | | | | | | |
| Activated alumina, “Alorco” | 200 | $N_2$ at-195° | — | — | — | 5 |
| Activated clay –6 | 147 | “ | 0.81 | 1.242 | 2.495 | 1 |
| Activated clay –7 | 223 | “ | 0.68 | 1.111 | 2.614 | 1 |
| Activated alumina desiccant | 175 | “ | 0.81 | 1.547 | 3.675 | 1 |
| Alumina, synthetic 10-30 mesh | 170 | “ | 0.88 | 1.58 | 3.23 | 10 |
| Asbestine pigment | 4.1 | $H_2O$ at 25° | — | — | — | 6 |
| Attapulgus clay | 170 | $N_2$ at-195° | — | — | — | 7 |
| Asbestos | 18 | “ | — | — | — | 8 |
| Basic carbonate white lead | 1.1 | $H_2O$ at 25° | — | — | — | 6 |
| Barytes pigment | 0.59 | “ | — | — | — | 6 |
| Barium sulfate | 0.43 | “ | — | — | — | 14 |
| Bauxite 8-14 mesh | 228 | $N_2$ at-195° | 0.88 | 1.575 | 3.622 | 1 |
| Bauxite, Arkansas I, 10-20 mesh | 289 | “ | 0.90 | — | — | 9 |
| Bauxite, Arkansas II, 10-20 mesh | 353 | “ | 0.90 | — | — | 9 |
| Bauxite, British Guiana, 10-20 mesh | 280 | “ | 0.90 | — | — | 9 |
| Bauxite, French, 10-20 mesh | 82.5 | “ | 1.30 | — | — | 9 |
| Bauxite, Arkansas 1, 10-30 mesh | 236 | $N_2$ at-195° | 0.97 | — | — | 10 |
| Bauxite, Arkansas 2, 10-30 mesh | 176 | “ | 0.93 | — | — | 10 |
| Bauxite, Arkansas 3, 10-30 mesh | 200 | “ | 0.86 | — | — | 10 |

TABLE 2 (*continued*)

| Material | Surface area | | Density gms./ml. | | | Reference |
|---|---|---|---|---|---|---|
| | m.²/gm. | Gas at °C. | Bulk | Particle | True | |
| Bauxite, South America, flaky 10-30 mesh | 187 | $N_2$ at-195° | 0.96 | — | — | 10 |
| Blanc fixe pigment | 2.2 | $H_2O$ at 25° | — | — | — | 6 |
| Bentonite, (<0.3 $\mu$) | 18.7 | $N_2$ at-195° | — | — | — | 11 |
| Brucine montmorillonite | 12.3 | “ | — | — | — | 12 |
| Clay | 10 | $N_2$ at-195° | — | — | — | 8 |
| Copper sulfate, anhydrous | 6.23 | “ | — | — | — | 16 |
| Chromium oxide gel | 228. | “ | — | — | — | 16 |
| ditto, sintered | 28.3 | “ | — | — | — | 16 |
| Diaspore, AlO(OH) | 3.9 | “ | — | — | — | 12 |
| Diatomaceous earth | 4.2 | “ | 0.32 | 0.631 | 2.265 | 1 |
| Diatomaceous earth | <1. | “ | 0.29 | 0.470 | 2.327 | 1 |
| Ferric oxide gel | 211 | $C_2H_5OH$ | — | — | — | 15 |
| Ferric oxide gel, water aged | 52 | “ | — | — | — | 15 |
| Fullers earth | 129. | $N_2$ at-195° | 0.55 | 0.860 | 2.660 | 1 |
| Giblisite, $Al(OH)_3$ | 0.31 | $N_2$ at-195° | — | — | — | 12 |
| Glass spheres, 12-micron | 0.216 | “ | — | — | — | 17 |
| Glass, sized beads (7.2 $\mu$) | 0.55 | “ | — | — | 2.237 | 14 |
| Glass, fritted, 8-14 mesh | 0.69 | “ | 0.87 | 1.608 | — | 1 |
| Glass, porous (Corning Glass Company) | 120 | “ | — | — | — | 13 |
| Glaucosil | 82 | “ | — | — | — | 16 |
| Halloysite | 43.2 | “ | — | — | — | 12 |
| Illite (hydrous mica, < 0.3 $\mu$) | 97.1 | “ | — | — | — | 12 |
| Kaolinite (< 0.3 $\mu$) | 15.5 | $N_2$ at-195° | — | — | — | 12 |
| Kieselguhr | 22.2 | “ | — | — | — | 18 |
| Lithopone pigment | 2.3 | $H_2O$ at 25° | — | — | — | 6 |
| Montmorillonite | 15.5 | $N_2$ at-195° | — | — | — | 12 |
| Montmorillonite | 11.2 | “ | — | — | — | 11 |
| Palladium catalyst | 0.35 | “ | — | — | — | 22 |
| Paper, insulating | 0.55 | “ | — | — | — | 28 |
| Paris white | 2. | “ | — | — | — | 8 |
| Porcelain, Coors | 1.6 | “ | 1.00 | 1.801 | 2.612 | 1 |
| Potassium chloride (< 200 mesh) | 0.24 | “ | — | — | — | 16 |
| Pumice | 0.38 | “ | — | — | — | 16 |
| Quartz, 400 mesh | 0.361 | “ | — | — | — | 17 |
| Quartz, flint, 4-6 mesh | <1. | “ | 1.53 | 2.610 | 2.641 | 1 |
| Silica gel | 620. | “ | — | — | — | 3 |
| Silica gel | 765 | “ | — | — | — | 3 |
| Silica gel I, non-electrolyzed | 584 | “ | — | — | — | 16 |
| Silica gel II, electrolyzed | 614 | “ | — | — | — | 16 |
| Silica gel | 866 | $N_2$ at-195° | — | — | — | 19 |
| Silica gel | 414 | “ | — | — | — | 20 |
| ditto + 5.2% $Al_2O_3$ | 541 | “ | — | — | — | 20 |
| Silica gel, 4-8 mesh | 669 | “ | 0.77 | 1.226 | 2.251 | 1 |
| Silica gel, 10-30 mesh | 602 | “ | 0.78 | 1.32 | 2.12 | 10 |

TABLE 2 (*continued*)

| Material | Surface area | | Density gms./ml. | | | Reference |
|---|---|---|---|---|---|---|
| | m.²/gm. | Gas at °C. | Bulk | Particle | True | |
| Silica aerogel V | 410 | $CCl_4$ | — | — | — | 21 |
| Silica aerogel V | 370 | $C_6H_6$ | — | — | — | 21 |
| Silica aerogel III | 330 | $H_2O$ | 0.92 | — | — | 21 |
| Silica aerogel | 393 | Butane, 0° | — | — | — | 15 |
| Silica aerogel | 690 | $N_2$ (—183°) | — | — | — | 15 |
| Silica aerogel, water-aged | 186 | Butane, 0° | — | — | — | 15 |
| Silica alumina gel, 4-8 mesh | 280 | $N_2$ at-195° | 1.00 | 1.558 | 2.369 | 1 |
| Silica alumina gel, 4-8 mesh | 404 | " | 0.75 | 1.175 | 2.388 | 1 |
| Silica alumina gel, 4-8 mesh | 467 | " | 0.69 | 1.126 | 2.377 | 1 |
| Silica alumina gel, 4-8 mesh | 372 | " | 0.64 | 0.962 | 2.378 | 1 |
| Silica alumina gel, 4-8 mesh | 409 | " | 0.55 | 0.860 | 2.402 | 1 |
| Silica alumina gel, 4-6 mesh | 323 | " | 0.55 | 0.932 | 2.405 | 1 |
| Silica alumina gel, 4-6 mesh | 201 | " | 0.68 | 1.050 | 2.343 | 1 |
| Titanium dioxide pigment | 8.2 | $H_2O$ at 25° | — | — | — | 6 |
| Titanium dioxide | 13.9 | $N_2$ at-195° | — | — | — | 27 |
| Titanium dioxide | 13.8 | $H_2O$ at 25° | — | — | — | 27 |
| Ultramarine blue pigment | 13 | " | — | — | — | 6 |
| Whiting pigment | 2.6 | " | — | — | — | 6 |
| Zinc oxide pigment | 2.4 | $H_2O$ at 25° | — | — | — | 6 |
| Zinc oxide, pigment K-1602 | 3.80 | $N_2$ at-195° | — | — | — | 14 |
| Zinc oxide, pigment F-1601 | 9.48 | " | — | — | — | 14 |
| Zinc oxide, pigment G-1603 | 3.88 | " | — | — | — | 14 |
| Zinc oxide, pigment KH-1604 | 0.658 | " | — | — | — | 14 |
| Zirconium orthosilicate | 2.76 | " | — | — | — | 14 |

±35 per cent and a relative accuracy of about ±5 per cent.[25] TABLE 2 contains a summary of the values for a large number of surface determinations which have been published in the last ten years. While this tabulation is not complete, it does demonstrate the keen interest initiated by the introduction of this new experimental tool.

The surface area available to nitrogen molecules may not necessarily be that available to larger molecules. Emmett and DeWitt[13] have shown, for example, that the surface areas of a porous glass as determined with nitrogen, argon, or oxygen are in agreement but that determined with butane was about 40 per cent lower. It seems reasonable to find that some parts of a porous adsorbent would be available to a small gas molecule and not to a larger molecule. Emmett,[25] however, has since indicated some justification for assigning to some adsorbate molecules larger cross-sectional areas than those calculated from liquid densities. For example, values of 11.3, 56.6, and 64 sq. Å for the water, *n*-butane, and *n*-heptane molecule, respectively, instead of 10.5, 32, and 45 sq. Å were found by Emmett to make the areas of crystalline solids calculated by Harkins and Jura agree with those calculated by the BET method. It is important to view these area determinations as a concept of surface available to the particular adsorbate molecule until more is learned about a greater variety of adsorption systems.

There are other properties besides density (bulk, particle, or true values) and surface area which are pertinent to a physical description of an adsorbent. Particle size is an important characterization. Zechmeister and Cholnoky[23] remark that the particle size of the column contents must not be too diverse, otherwise the finer particles may act almost like a different adsorbent. The particle size distribution may influence three important experimental quantities: (1) rate of flow; (2) channeling through column; and (3) sharpness of the boundaries separating zones. The dependence of the rate of flow on particle size is well known. Channeling is due to the presence of a path of least resistance to flow, and the chance of realizing such behavior is greater when the particle size distribution is broad. The reproducible packing of an adsorption column becomes a simpler operation when the particles are of uniform size. Variation in the shapes of the particle tend to cancel out on the average. It is apparent that the closer the kinetics of flow are to simple piston-displacement, the sharper may be the boundaries separating the zones. A closer control of particle size distribution is one factor to which the manufacturers of adsorbents could contribute in order to realize the standardization of materials for use in chromatography.

Relatively little progress has been made on a correlation of the chemical properties of various preparations of adsorbents. A chemical analysis of the surface layer may be only approximated from the complete chemical analyses of the adsorbent itself. In some special cases, the structure of the adsorbing surface has been worked out in detail.

In solution adsorption phenomena, it is probable that the adsorption is confined to monolayers. All components of the solution compete for the available adsorbing surface. In dilute solutions, for example, it is likely that the solvent is adsorbed in great excess to the solute. It is very desirable to know the absolute amounts of all adsorbed components, and this constitutes an outstanding problem in the present studies of adsorption from solutions.

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# CONCLUDING REMARKS

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The foregoing papers are a partial record of the Conference on Chromatography held by The New York Academy of Sciences. The record is a partial one for two reasons. Firstly, it has been impossible to include an interesting paper on *Chromatography in the Isolation and Separation of the Various Penicillins* by Dr. Jacques L. Wachtel, though Dr. Wachtel was able to speak freely on this subject and to hold some discussion of it. Secondly, the record is a partial one because no written words can do justice to the discussion, to the stimulation of new ideas, or to the clarifications of old problems which come from such a meeting.

In gathering the material for this monograph, the Chairman, with the full support of the Editors, has sought partially to redress the second source of incompleteness mentioned above. It was apparent from the discussion of papers that at least three additional topics needed attention. It seemed appropriate to invite Dr. Victor R. Deitz to write a paper in which data characterizing solid adsorbents of possible use in chromatography were gathered into handy, usable form. This paper should facilitate a quantitative approach to certain of the problems of chromatography.

Ion-exchange had not been brought formally under the scrutiny of the Conference, yet it is certainly assuming increasing importance as a tool which may be applied chromatographically. Accordingly, at the suggestion of one of the Editors, Mr. Norman Applezweig was invited to write the paper reviewing this field. It is hoped that this paper will aid in bringing before a wider audience the natural and synthetic exchange substances.

The non-uniformity of adsorbent surfaces was a subject of uniform concern and frequent discussion at the Conference. It therefore seemed proper to the Chairman to invite Dr. Leo Shedlovsky to contribute a paper on separations using foams and emulsions. The surfaces involved in mobile interfaces are likely to be of a sameness throughout the system, and, thus, to meet some of the requirements hoped for in the discussion and be worthy of further study. Moreover, while chromatography has concerned itself almost entirely with solid-liquid or solid-gas interfaces, it has seemed to the Chairman that liquid-liquid or liquid-gas interfaces should also come into consideration here. The experiments of Schütz and others have now shown that interesting separations can be obtained by the counter-current application of partition between the liquid-gas interfacial and bulk liquid phases. This paper by Dr. Shedlovsky may stimulate chromatographers to further investigation of the mobile interfaces.

It is perhaps worth while to report the general pattern which seemed, to the Chairman, to run through the Conference.

Chromatography, which was first devised as a tool applied empirically, has in the last decade or so become better understood. With understanding has come the flourishing development described by Dr. Zechmeister in his first paper. We are in the process, now, of further understanding and sharpening not only the classical Tswett chromatography but also the elegant simplifications introduced by Tiselius and Claesson, and the ingenious and elegant "partition chromatography" of Martin and Synge.

It is probably correct to say that, while it may look as though some ultimate point has been reached in the refinement and subtlety of chromatography as these have been revealed in the foregoing papers, yet experience allows us to prophesy that there will be no end to progress. It is quite reasonable to expect that some of those who attended the Conference or will read these papers will be stimulated to important new ideas.

One area of investigation in which there is evident need for continued research and inspiration—continued, because research and inspiration are immanent in the foregoing papers—is that which connects adsorbability with chemical or physical properties of molecules. It was implied, in the discussions, that we should expect no simple relationship to be found here. Yet I venture to wonder if we might not, through some principle of exclusion, find a simple set of relations. We may find that, under given circumstances, a large proportion of the factors which might theoretically add complexity to this relationship drop out instead, leaving only one or two controlling factors to be dealt with.

A basis for such a hope as this may be found in the work of Tiselius and his group. Here, in the face of the complexities of chromatographic theory so well delineated by Dr. Thomas, simplifications were wrought which have brought the theory under practical control. An example of such simplification is the use of the displacement developer, which with one stroke can control the behavior of the rest of the adsorbates in the column. Perhaps, with this precedent, we can look for further simplifications, whether in technique or theory or in relations which connect structure and properties of molecules with their behavior in the column.

The need for standardized adsorbents, which was stressed especially by Dr. Zechmeister, has already been mentioned.

Those who attended the Conference and who will read these pages are indebted to the authors. They gave freely of their time, and, in some cases, came from far away to contribute to the success of the Conference and the existence of this monograph. Theirs was a real act of faith in the objective of these conferences: The free interchange of scientific knowledge and ideas among scientists. The spirit which animated the Conference was in accord with the advice given by Bacon long ago: "We advise all men to think of the true ends of knowledge, and that they endeavor not after it for curiosity, contention, or the sake of despising others, nor yet for reputation or power or any other such inferior consideration, but solely for the occasion and uses of life."

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